Evidence of the Involvement of O-GlcNAc-modified Human RNA Polymerase II CTD in Transcription in Vitro and in Vivo*

Received for publication, December 5, 2011, and in revised form, May 10, 2012. Published, JBC Papers in Press, May 17, 2012, DOI 10.1074/jbc.M111.330910

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Background: Modifications of the RNA polymerase II CTD are necessary for transcriptional regulation.

Results: Perturbation of O-GlcNAc addition and removal showed transcription defects in vitro and in vivo.

Conclusion: O-GlcNAc modification of the CTD functions in transcription initiation.

Significance: These data provide an additional modification of the CTD that acts before the initiation of transcription.

The RNA polymerase II C-terminal domain (CTD), which serves as a scaffold to recruit machinery involved in transcription, is modified post-translationally. Although the O-GlcNAc modification of RNA polymerase II CTD was documented in 1993, its functional significance remained obscure. We show that O-GlcNAc transferase (OGT) modified CTD serine residues 5 and 7. Drug inhibition of OGT and OGA (N-acetylglucosaminidase) blocked transcription during preinitiation complex assembly. Polymerase II and OGT co-immunoprecipitated, and OGT is a component of the preinitiation complex. OGT shRNA experiments showed that reduction of OGT causes a reduction in transcription and RNA polymerase II occupancy at several B-cell promoters. These data suggest that the cycling of O-GlcNAc on and off of polymerase II occurs during assembly of the preinitiation complex. Our results define unexpected roles for both the CTD and O-GlcNAc in the regulation of transcription initiation in higher eukaryotes.

Eukaryotic RNA polymerase II has a unique structure at its C terminus, the C-terminal domain (CTD). The CTD in humans consists of 52 imperfect repeats of the consensus sequence YSPTSPS (1, 2), with serines 2, 5, and 7 phosphorylated subsequently to transcription initiation (3, 4). Current models state that two species of RNA polymerase II (pol II) exist: unphosphorylated (pol IIA) and phosphorylated (pol IIO) (5). In vitro transcription assays showed that pol IIA was incorporated into preinitiation complexes (PICs) that form on promoter DNA. Pol IIO forms upon the initiation of transcription and defines the elongation-specific form of pol II (6–10). However, neither state of the CTD affects the intrinsic catalytic activity of pol II, and in vitro, in purified systems, a CTD-less pol II is capable of directing transcription (11–14). Rather, CTD phosphorylation serves to recruit various post-transcriptional mRNA processing factors to the elongating polymerase (2). The CTD likely also has a role during the initiation of transcription, because of the defects seen in initiation using truncated CTDs (15, 16) and the functional and physical interactions of the Mediator coactivator complex with the CTD (17–22).

However, other work suggests that evidence that pol IIA is the form of pol II entering the PIC should be reconsidered. Kelly et al. (23) show that “unmodified” pol II (pol IIA), (purified from calf thymus as in Ref. 7) contains a population of pol II that is O-GlcNAcylated on the CTD. O-GlcNAc (N-acetylglucosamine) is a post-translational modification added to serine and threonine residues via an O-linkage by O-GlcNAc transferase (OGT) and UDP-GlcNAc (24). Removal of O-GlcNAc is catalyzed by the N-acetyl-β-d-glucosaminidase (OGA) (25). Edman degradation indicates that the threonine at position 4 and the serine at position 5 of the CTD are O-GlcNAcylated (23). There is no O-GlcNAcylation of the elongation-specific phosphorylated CTD (RNA polymerase IIO). Secondarily, O-GlcNAc-modified CTD peptides are refractory to further phosphorylation by the Ser-5-specific CTD kinase CDK7, a component of the general transcription factor TFIIH, consistent with the proposal that O-GlcNAc and phosphorylation are often mutually exclusive (26). However, the functional consequences of O-GlcNAc modification of the CTD and whether the O-GlcNAc modification of the CTD has a role in transcription have not been determined.

These data suggest that it is not clear which form of pol II is entering the PIC, as the experiments in Chesnut et al. (7) do not distinguish between a true unmodified pol II and O-GlcNAc-modified pol II. We therefore have attempted to discern the functional significance of GlcNAc-modified RNA
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pol II. We provide several lines of evidence that pol II is O-GlcNAc-modified and that pol II O-GlcNAcylation occurs in vitro and in vivo. These data suggest that pol II undergoes a cycle of O-GlcNAcylation at the promoter during assembly of the preinitiation complex, which is required for promoter activity and/or regulation. Furthermore, these data point to O-GlcNAc-pol II as being an initiation-specific pol II species.

**EXPERIMENTAL PROCEDURES**

**Reagents**—The reagents used were STO45849 (Tim Tec), allooxan (Sigma), 8WG16 (Covance), anti-OGT AL28 or AL24 (Hart laboratory), anti-TBP 3G3 mAb (Chemicon), Dynabeads (Invitrogen), UDP-GlcNac (Sigma), 110.6 (Hart laboratory), E3 promoter DNA (27), and anti-pol II N20, A20, and F12 (Santa Cruz Biotechnology). Anti-TFIIA (rabbit), anti-TFIIF (rabbit), and anti-TFIH mAb were provided courtesy of D. Reinberg. O-(2-Acetamido-2-deoxy-α-D-glucopyranosylidene)n-phosphorylcarbamate (PUGNAC) and 1,2-dideoxy-2′-methyl-α-D-glucopyranosyl(2→3)-2′-thiazoline (GlcNac-thiazoline (NAGT)) were synthesized by the Medicinal Chemistry Core Facility at Johns Hopkins University. Wheat germ agglutinin (WGA) resin (Vector Laboratories), protein gel/MOPS buffers (Invitrogen), H5/H14 (Covance), TFIIF (ProteinOne), rP-TEFb (ProteinOne), anti-GST antibody (Santa Cruz), and Complete protease inhibitors (Roche Applied Science) were also used.

**In Vitro Transcriptions**—Assays were as described (28). Briefly, HeLa nuclear extracts (25–50 μg) were incubated with 0.5 mM NTPs and 0.1 μg of supercoiled promoter DNA for 30 min. RNA was extracted and used in primer extension assays with 32P-end-labeled primers. 10% acrylamide/TBE/urea gels were used to separate RNA products, which were visualized by autoradiography. Human RNA polymerase IIIA was supplied by the laboratory of D. Reinberg (NYU School of Medicine). Inhibitor concentrations were: STO45849, 0.05–0.4 mM; PUGNAC, 0.2–4 mM; pol IIA add-back experiment (Fig. 5, D and E, 4 mM). In Figs. 2 and 3, C and D, inhibitors were added concomitantly with NTPs. In Figs. 3, C and D, an equivalent amount of either pol II A or pol II γ was added to PUGNAC-treated nuclear extract.

**Immobilized Templates**—Assays were carried out by incubating 0.5 μg of biotinylated E3 promoter DNA (created by PCR using a biotinylated T7 primer for the upstream primer and SP6 primer) with 10 μl of M280 Dynabeads as per the manufacturer’s instructions. The beads were then incubated for 1 h with 50 mg/ml BSA. The beads were briefly washed in H.1 buffer (20 mM Hepes, pH 7.9, 100 mM KCl, 0.2 mM EDTA, and 10% glycerol) and incubated with 50 μl of HeLa nuclear extract, 75 μl of HM.1 buffer (H.1 buffer plus 12.5 mM MgCl₂), and 125 μl of H₂O plus 2 μg of Escherichia coli DNA (determined by titration) at room temperature for 30 min. Afterward, the beads were washed three times for 20 min each with H.2, 0.05% Nonidet P-40. Bead pellets were then heated in sample buffer, run on a 4–12% gradient SDS-PAGE, and transferred overnight to nitrocellulose. PUGNAC and allooxan inhibitors were added concomitantly with the DNA and HeLa nuclear extract (PUGNAC at 4 mM final; allooxan at 0.5 mM final).

**Recombinant Protein Purification**—rOGT, rOGA, and rGST-CTD bacterial expression vectors were transformed into BL21(DE3). Cells were grown to and OD of 0.4 to 0.6 and induced with 1 mM IPTG for 3 h at 37 °C. Cells were resuspended in PBS containing 1% Nonidet P-40, 1 mM EDTA, and Complete protease inhibitors (Roche Applied Science) and lysed by sonication. rOGT, rOGA, and GST-CTD (and CTD mutants) were purified using standard nondenaturing protocols. rOGT and rOGA were purified after sonication and clarification over a nickel-nitrilotriacetic acid-Sepharose Hitrap column using an AKTA purification system (GE Healthcare). Bound proteins were eluted with a 50–250 mM imidazole gradient. Detection was first done using A₂₈₀, elution profiles and confirmed by SDS-PAGE. GST-CTD proteins were purified over GT-Sepharose Hitrap columns and eluted with a glutathione gradient. All proteins were aliquoted and frozen at −80 °C.

**Enzymatic Reactions**—For OGT, 3 μg of GST-CTD (and mutants) or 1 μl of RNA pol II, 1 μl (1.5 μg) of rOGT, 5 mM UDP-GlcNac, 12.5 mM MgCl₂, and 50 mM Tris, pH 7.4, were used; the reaction was at 37 °C for 30 min (29). The OGA assay was performed essentially as described (30). P-TEFb labeling of GST-CTD was as described (31). TFIH kinase assays contained 3 μg of GST-CTD or GST-CTDγ plus partially purified TFIH fraction and 1 mM ATP under the buffer conditions used for the OGT reactions above.

**Western Blots**—Western blot assays were performed using nitrocellulose filters (Whatman, 0.45 μm) and Western transfer buffer (Invitrogen). Polyacrylamide gels were either 10% or 4–12% gradient gels (MOPS buffer system, Invitrogen). Polyacrylamide gels were either 10% or 4–12% gradient gels (MOPS buffer system, Invitrogen). Western blots were developed with the appropriate primary and secondary antibodies (anti-mouse IgM-HRP, Santa Cruz Biotechnology) and detected by ECL (Pierce). Western blots with the 110.6 mAb were done as described (32). All other Western blots were treated with standard protocols, blocked with either 5% milk/Tris-Tween or 3% BSA/Tris-Tween and washed with Tris-Tween buffer.

**Sugar Nucleotide Determination**—Nuclear extracts were lyophilized and extracted with 0.75 ml of cold 0.5 N perchloric acid. The suspension was dispersed vigorously for 20 s in an ice bath followed by centrifugation at 15,000 × g for 10 min in a cold room, and the supernatant was collected. The pellet was re-extracted similarly, and both supernatants were pooled. 200 μl of charcoal suspension (30 mg of Mallinckrodt charcoal/ml of 1 N perchloric acid) was added to the cold supernatant and stirred vigorously in an ice bath. After centrifugation as described above, the supernatant was discarded. The charcoal pellet was eluted three times with 750 μl of a solution containing 50% EtOH and 1% NH₄OH. After the ethanol was removed, the supernatant was frozen and lyophilized. The bound nucleotide fraction was hydrolyzed and analyzed by Dionex HPLC using a PA10 anion exchange column and pulsed amperometric detection as described previously (33).

**Chromatin Immunoprecipitation (ChIP)**—A total of 30 million BJAB cells were treated with formaldehyde (37%) to a final concentration of 1% added directly to the culture medium for 15 min at room temperature with gently shaking. Cross-linking was stopped by adding glycerol to a final concentration of 0.125 M for 10 min at room temperature with gentle shaking. Cells were harvested by spinning down at 1300 rpm for 5 min. After two washings in PBS, cells were resuspended in lysis buffer (50
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FIGURE 1. In vitro analysis of the O-GlcNAc modification of human RNA polymerase II. A, depiction of silver-stained SDS-PAGE of purified human RNA polymerase II used in the experiments herein and purified as per Maldonado et al. (55). B, shown is a Western blot analysis of the modification of RNA polymerase II with O-GlcNAc using the anti-O-GlcNAc antibody 110.6 (26). The O-GlcNAc is added by incubating purified RNA polymerase II with rOGT and UDP-GlcNAc. Two independent reactions of pol II with OGT are shown. The lower panel shows pol II incubated with the CTD-specific 8WG16 mAb as a loading control (assayed with 110.6 anti-O-GlcNAc antibody), or pol II incubated with His-OGT and His-TBP were Co-immunoprecipitated with anti-His antibody (Santa Cruz Biotechnology) and developed with an anti-GST antibody. C, O-GlcNAc-modified Rpb1 subunit of RNA pol II can be detected in nuclear extracts. Rpb1 was detected by Western blot using an antibody recognizing the N terminus of Rpb1. Lane 1, purified human RNA pol II Western blot control; lane 2, immunoprecipitations of HeLa NE using the 110.6 anti-O-GlcNAc IgM mAb plus anti-mouse IgM-agarose beads; lane 3, same immunoprecipitation as in lane 2 with the addition of the OGA inhibitor PUGN Ac (4 mM); lane 4, control immunoprecipitation using only anti-mouse IgM-agarose beads incubated with HeLa NE. D, isolation of O-GlcNAc-modified RNA pol II from HeLa NE using WGA-agarose beads. Lanes 1 and 2 show preparations with WGA beads, whereas lane 3 shows pol II incubated with His-OGT. Lanes 1 and 2 show preparations with His-OGT, whereas lane 3 shows pol II incubated with His-OGT. E, STO45849 OGT inhibitor blocks O-GlcNAc modification of RNA pol II in nuclear extracts. HeLa NE were incubated as described in D with either PUGN A (4 mM (lane 2)), STO45849 (0.4 mM STO (lane 3)), or both inhibitors (lane 4). O-GlcNAc-modified proteins were purified with WGA-agarose, eluted in sample buffer, and run on 10% SDS-PAGE. Western blots were done with an anti-N-terminal Rpb1 antibody (F12, Santa Cruz Biotechnology). F, comparison of the migration of unmodified native pol II (pol IIA; N-terminal antibody), pol IIA (assayed with 110.6 anti-O-GlcNAc antibody), or pol IIO (assayed with the HS antibody specific for CTD serine 2 phosphorylation) on SDS-PAGE. The resulting Western blots were developed separately and overlaid relative to the molecular weight standards to more easily visualize the mobility differences. pol IIA is the same material as used in A, pol IIA was made as described in B, pol IIO was made with rP-TEFB and ATP. G, comparison of the relative migrations of GST-CTD and O-GlcNAc-modified GST-CTD in SDS-PAGE. Lanes 1 and 2 show a Western blot of GST-CTD (lane 1) and GST-CTD incubated with rOGT and UDP-GlcNAc (lane 2). The blot was developed with the O-GlcNAc-specific 110.6 antibody. Lanes 3 and 4 are GST-CTD or O-GlcNAc-modified GST-CTD as shown in lane 2. The Western blot (WB) seen in lanes 3 and 4 was developed with an anti-GST antibody. H, analysis of pol II as a substrate for OGA. Two isoforms of OGA (OGA and OGAα) were assayed using pol II (made as described in B) at two different pH levels, 6.4 and 7.4. Loss of O-GlcNAc was assayed by SDS-PAGE and Western blot following incubation with OGA. Blots were assayed with the 110.6 a-O-GlcNAc antibody (upper panel) or with the CTD-specific 8WG16 mAb as a loading control (αRpb1 blot (lower panel)).

Oxidized antibody with incubation for 30 min at room temperature.

Co-Immunoprecipitations—25 μg of protein G-agarose beads (Roche Applied Science) was mixed with 10 μg of antibody or the corresponding normal mouse and/or normal rabbit IgG control and incubated for 3 h at 4 °C with rotation. HeLa nuclear extract (25 μg) was added to the antibody-beads mixture and incubated overnight with rotation at 4 °C. Beads were washed in HM.1 buffer supplemented with 0.1% Tween 20 twice for 15 min each by rotation at 4 °C. Beads were resuspended in loading buffer and incubated at 75 °C for 5 min. Proteins were separated on 4–12% gradient SDS-polyacrylamide gels and transferred overnight at room temperature for pol II and at 45 v for 3 h at room temperature for the other proteins.

Co-immunoprecipitations of His-OGT and His-TBP were done in BC100 (20 mM Tris, pH 7.9, 100 mM KCl, 0.2 and mM EDTA) for 30 min prior to incubation with 3G3 anti-TBP mAb and protein G-agarose beads. Beads were washed in BC100, heated in sample buffer, and run on 10% SDS-PAGE. After transferring to nitrocel lulose (Whatman), blots were probed with anti-His antibody (Santa Cruz Biotechnology) and developed by ECL (Pierce).

For wheat germ agglutinin affinity purification, 20 μl of WGA-agarose slurry was incubated with 100 μl of HeLa nuclear extract (8 mg/ml) plus 4 mM PUGNAc for 2 h at 4 °C. Beads were washed in H.1 buffer and analyzed by SDS-PAGE and Western blot.

RESULTS

Human RNA Polymerase II Is a Substrate of Both OGT and OGA—Previous work established that CTD peptides were substrates for OGT (26). We first wished to establish that native human RNA polymerase II is in fact a substrate for the O-GlcNAc-modification enzymes. We purified rOGT from bacterial extracts and used that to modify highly purified human RNA polymerase II (Fig. 1A). We assayed for O-GlcNAc...
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by Western blot using 110.6, an anti-O-GlcNAc antibody raised against synthetic O-GlcNAcylated CTD peptides (35). This resulted in a significant O-GlcNAc signal in two separate OGT reactions, indicating the O-GlcNAc modification of the CTD (Fig. 1B).

We next asked whether endogenous RNA polymerase II (pol II) in nuclear extracts was O-GlcNAcylated. We assayed this in two ways. In the first, we treated HeLa nuclear extracts with the OGA inhibitor PUGNAc (to stabilize any O-GlcNAc on pol II) and immunoprecipitated with 110.6. The immunoprecipitate was analyzed by Western blot using the Rpb1 N-terminal-specific antibody N20. We were able to detect O-GlcNAc-pol II in this manner (Fig. 1C). Our second approach used WGA-agarose beads to affinity purify O-GlcNAc-modified proteins from the nuclear extract (32). Again, these products were assayed in duplicate with the N-terminal pol II antibody N20 by Western blot (Fig. 1D, compare lane 1 with 2 and 3 with 4). Here we detected a slower migrating form of pol II that immunoprecipitated only with the addition of PUGNAc.

Furthermore, the nuclear extracts had the ability to modify RNA pol II with O-GlcNAc. We measured the levels of UDP-GlcNAc in the nuclear extracts and determined that the concentration was 220 ± 14 (three determinations ± 2 S.D.) μmol/mg protein (data not shown; see “Experimental Procedures”). This value is consistent with a concentration of ~0.5 μM endogenous UDP-GlcNAc and is very similar to the OGT Km for UDP-GlcNAc, meaning that the nuclear extract [UDP-GlcNAc] is within the operational window of OGT (36, 37). Lastly, we incubated nuclear extracts with STO45849, an inhibitor of OGT (Gross et al. (43)), and purified O-GlcNAc-modified proteins with WGA-agarose. Nuclear extracts incubated with STO45849, PUGNAc, or both were compared by Western blotting for pol II. We again observed the increase in O-GlcNAcylated pol II in the presence of PUGNAc (Fig. 1E, compare lanes 1 and 2). We did not see this increase with STO45849, and most importantly, STO45849 blocked the increase in O-GlcNAcylated pol II that we obtained using PUGNAc alone (Fig. 1E, compare lanes 2 and 4). This indicates that the STO45849 blocked OGT activity in the nuclear extracts.

We next compared the mobility of the three forms of pol II using phospho- and O-GlcNAc-specific antibodies in Western blots (Fig. 1F). To best show these small differences, we overlaid the results of the Western blots with the three different antibodies relative to the molecular weight markers. We consistently observed that O-GlcNAc-pol II migrated as an intermediate species, in between unmodified pol IIa and P-TEFb-phosphorylated pol IIO. We refer to the O-GlcNAc-modified form of RNA pol II as pol IIy. To further support this mobility difference, we performed the same OGT assay using GST-CTD (containing 26 heptad repeats) as a substrate. As seen in Fig. 1G (compare lanes 1 and 2), the GST-CTD was modified by OGT. Furthermore, when comparing the mobility differences between GST-CTD and O-GlcNAc-modified GST-CTD using an anti-GST antibody, we found the O-GlcNAc-modified GST-CTD had a reduced mobility in SDS-PAGE (Fig. 1G, compare lanes 3 and 4). These data are consistent with the determination of sites that can be modified by OGT; the CTD heptad repeat is clearly contained within the derived consensus sequence of sites within proteins that are O-GlcNAcylated (38).

We also asked whether native human pol IIy was a substrate for OGA. We assayed two isoforms, the full-length OGA and the 75-kDa short isoform, OGAs (39, 40). We assayed for N-acetylglucosaminidase activity using two different pH levels and buffers to optimize OGA activity: pH 6.5 to reproduce conditions of its detection and pH 7.4 to assess a more physiological pH. Following incubation of pol IIy with either of the OGA isoforms, we assayed for the presence of O-GlcNAc by Western blot (Fig. 1H, upper panel). With both isoforms we obtained almost complete removal of O-GlcNAc from native RNA pol IIy, although the full-length OGA was more active at pH 7.4 than at 6.4 (and vice versa for OGAs). In any case, pol IIy, derived from native human pol II, is a legitimate substrate for OGA in vitro. In all cases we did not see any loss of Rpb1 itself, indicating that our loss of O-GlcNAc was not due to a contaminating protease (Fig. 1H, lower panel).

OGT- and OGA-specific Inhibitors Abrogate Transcription in Vitro—To address the functional role of pol IIy, we began to study O-GlcNAc functions in transcription by utilizing a cell-free transcription system (27, 28, 41, 42). We took advantage of the existence of several inhibitors of both OGT and OGA and asked whether such inhibitors would have transcription defects in our cell-free system. Indeed, STO45849 and PUGNAc, inhibitors of OGT and OGA, respectively (43) (25, 44), significantly blocked transcription from the adenovirus E3 promoter, so chosen because it contains a representative and typical core promoter architecture and is robustly transcribed in vitro (Fig. 2, B and C) (27). We found similar results when assaying a second promoter containing the DPE-class core promoter element (Fig. 2D) (28, 45, 46). In addition to STO and PUGNAc, alloxan, a dual OGT and OGA inhibitor, and 1,2-dideoxy-2′-methyl-α-d-glucopyranosyl-[2,1-d]-Δ 2′-thiazone (NAGT), a second, more selective OGA inhibitor, also abrogated transcription in vitro (Fig. 2D) (47–49). Because we used different core promoter types, we inferred that the O-GlcNAc requirement in transcription is not restricted to one core promoter class and is potentially a widespread phenomenon.

OGT and OGA Function during PIC Assembly—These data suggest that the inhibition of O-GlcNAc cycling by OGT and OGA inhibitors causes a defect in transcription. We further tested this hypothesis to ask when during the transcription process the inhibitors act. Excluding ribonucleotides from the assay separates the in vitro transcription system into two steps. The first step, the assembly of the PIC onto the promoter DNA, is done simply by mixing nuclear extract and promoter DNA. After this assembly step, the addition of NTPs initiates transcription. We assayed for a defect in PIC assembly by incubating the OGT inhibitor, STO45849, or the OGA inhibitor, PUGNAc, with nuclear extracts either before or after a 30-min incubation of nuclear extract with E3 promoter DNA, i.e. before or after PIC assembly (Fig. 2E). We found that the addition of either STO45849 or PUGNAc before PIC assembly resulted in the complete abrogation of transcription. However, if PIC assembly occurred first, the subsequent addition of either inhibitor did not significantly decrease transcription from the E3 promoter (Fig. 2F) nor did a 30-min mock incubation have
any effect on transcription (Fig. 2G). These results show that both the addition and removal of O-GlcNAc occur before or during PIC assembly and before the initiation of transcription. If this was an early elongation defect, then we would expect to see transcription decrease regardless of when the inhibitors were added, because the NTPs were always added afterward.

**OGT Is Stably Recruited to the PIC and Associates with RNA Polymerase II**—Given the functional associations between OGT and pol II, we asked whether we could detect OGT as part of the PIC of the adenovirus E3 promoter. We assayed this via an immobilized DNA template of either the E3 promoter or non-promoter DNA, represented by the multiple cloning site (MCS) of the parent vector of the E3 promoter template, pSP72. Biotinylated E3 promoter DNA was bound to strepavidin-magnetic beads and incubated with HeLa nuclear extracts. After a 30-min incubation period, the bound proteins were eluted in sample buffer and analyzed by SDS-PAGE and Western blot (Fig. 3A). We consistently observed higher than background levels of pol II and TBP, both of which would be expected to be recruited to the E3 promoter. Likewise, we found that OGT was recruited to the promoter above the background levels seen when using MCS DNA. Although we used similar amounts of each DNA, the MCS DNA was ~3–4-fold more concentrated on a molar basis, because of its smaller size (Fig. 3A, right panel). Therefore these background protein levels are probably an overestimate.

Because the PUGNAc inhibitor blocks the removal of the O-GlcNAc from the CTD by the OGA, we reasoned that the addition of the unmodified form of pol II (pol IIA, purified from HeLa cells) would bypass this block (Fig. 3B). This experiment also served as a very important control; if the removal of O-GlcNAc from another protein was necessary for transcription, and if this removal was blocked in this assay by PUGNAc, then one would not expect the pol IIA to bypass the PUGNAc block in transcription. We found that titration of purified pol IIA resulted in the alleviation of the PUGNAc-mediated block in transcription (Fig. 3C).

A second prediction was that O-GlcNAc-modified pol II would not bypass the PUGNAc block. To test this, we first modified purified RNA polymerase II with rOGT and UDP-GlcNAc (Fig. 1B). We then titrated an equivalent amount of either pol IIA or pol IIγ into the *in vitro* transcription system containing PUGNAc. In contrast to the pol IIA titration, the pol IIγ did not rescue the PUGNAc block to transcription (Fig. 3D). Titration of rOGT and UDP-GlcNAc had no effect (data not shown). These results suggest that RNA polymerase II is functionally the target of the PUGNAc block and that removal of O-GlcNAc from pol II is necessary for transcription to occur.

**Serines 5 and 7 of the CTD of RNA Polymerase II Are Necessary for OGT Activity**—The human CTD consists of 52 repeats of the heptad consensus sequence YSPTSPS. Kelly et al. (23) determined by Edman degradation of calf thymus RNA pol II...
that both Thr-4 and Ser-5 of the CTD are O-GlcNAc-modified. We wished to corroborate this result by assaying a panel of CTD alanine substitution mutants (kindly provided by Shona Murphy). The appropriate GST-CTD (containing ~26 heptad repeats) proteins were incubated with rOGT and UDP-GlcNAc and separated by SDS-PAGE. Western blots of the reaction products show that serine residues 5 and 7 specifically are necessary for OGT activity (Fig. 4A). Because alanine substitutions formally do not distinguish between a residue being modified and a requirement for that residue for substrate modification, we could not rule out the latter. However, given the Edman degradation results that serine 5 is O-GlcNacylated (23), these results are likely because serine 5 is modified directly by OGT. Secondly, it is well known now that serine residues are particularly labile in Edman reactions (50). Thus, it is likely that the amount of serine O-GlcNAcylation in the CTD in the findings of Kelly et al. (23) is an underestimate, whereas the O-GlcNAc on threonine might be an overestimate. It is apparent that either both of the serine residues are O-GlcNAcylated or that one residue is required for the accurate modification of the other, because both alanine substitutions completely abrogated O-GlcNAcylation. It is also possible that O-GlcNAcylation at serine 5 or 7 may be required for subsequent O-GlcNAcylation of threonine 4. Lastly, as a control for the integrity of the SSA and S7A CTD mutants, we assessed their efficacy as a substrate for the serine 2-specific CTD kinase, P-TEFb. In the experiment presented in Fig. 4B, we clearly observe that both mutants are efficiently phosphorylated, whereas the S2A mutant is not phosphorylated. Thus, the results obtained with OGT and the Ser-5 and Ser-7 mutants are likely because of the alanine substitutions themselves and not the nonfunctional substrates.

Serine residues 5 and 7 are sites of CTD phosphorylation by the general transcription factor TFIIH, in which activity is comitant with the initiation of transcription (4). Because there is an overlap between CTD residues modified by OGT and TFIIH, we asked whether O-GlcNAc-modified CTD would interfere with TFIIH phosphorylation of the CTD. We used GST-CTDY as a substrate for TFIIH and assayed for phosphorylation by Western blot using the serine 5-specific antibody H14 (Fig. 4C). As with Comer and Hart’s (26) results using rCAK and CTD peptides, we found that native TFIIH did not phosphorylate GST-CTDY as well as unmodified GST-CTD. These data indicate that O-GlcNacylation and serine 5 phosphorylation are mutually exclusive events.

Co-immunoprecipitations Reveal Interactions between OGT and the General Transcription Machinery—Given the data showing that OGT was present in a PIC, we asked whether RNA polymerase II and other general transcription factors (GTFs) interacted with OGT. Such information would shed light on how OGT is recruited to the PIC. To do these experiments, we immunoprecipitated pol II, TFIIA, TBP, the RAP74 subunit of TFIIF, and the ERCC3 subunit of TFIIH. We then probed these samples on immunoblots using an OGT antibody (Fig. 5). We found three antibodies to pol II that co-immunoprecipitated OGT, whereas the normal mouse IgG did not (Fig. 5A). We also found that anti-TFIIA, anti-TBP, and anti-TFIIH (ERCC3 subunit) antibodies co-immunoprecipitated OGT (Fig. 5, B, C, and E). The only GTF co-immunoprecipitated with the OGT antibody was the RAP74 subunit of TFIIF and perhaps ERCC3 (Fig. 5, D and E). We also did not observe any interactions of OGT with the FCP1 CTD phosphatase (Fig. 5F). Because these are crude nuclear extracts, it is likely that multiple interactions...
Figure 4. OGT modifies the CTD at serine positions 5 and 7. A, GST-CTD or GST-CTD containing single alanine substitutions at serine positions 2 or 5, a threonine to alanine substitution at position 4, or a double substitution at positions 2 and 5 of the CTD (each containing 26 heptad repeats and each confirmed by sequencing). The lower panel is an example of the loading of the GST-CTD proteins in each lane as assayed by anti-GST antibody. The upper panel shows the results of incubations of the CTD substrates with rOGT and UDP-GlcNAc. The O-GlcNAc modification was detected using the 110.6 anti-O-GlcNAc antibody. B, control phosphorylation reactions with P-TEFb kinase and the GST-CTD or GST-mutant CTDs as described in A. Phosphorylation was detected by Western blot with the anti-phosphoserine 2 antibody H5. The lower panel indicates the loading of each protein as indicated in an anti-GST Western blot. C, O-GlcNAc-modified CTD was assayed for its efficiency as a substrate for TFIIH phosphorylation. GST-CTD or O-GlcNAc-modified GST-CTD (GST-CTDγ) was incubated with native TFIIH and ATP, and phosphorylation was assayed by Western blot with the serine 5-specific H14 antibody. The bottom panel illustrates the loading of equivalent amounts of GST-CTD as assayed by an anti-GST antibody. GST-CTDγ was produced with rOGT and UDP-GlcNAc.

Figure 5. RNA polymerase II and several GTFs bind OGT in nuclear extracts. A, co-immunoprecipitations (IPs) were done using a panel of mAb against RNA polymerase II (A20, F12, and BWG16) and compared with a control immunoprecipitation using normal mouse IgG serum. The immunoblots were then assayed for either pol II or OGT. B, co-immunoprecipitations of TFIIA (mAb) and OGT (rabbit polyclonal) were compared with normal mouse or rabbit IgG (mIgG and rIgG, respectively). Immunoblots were probed for TFIIA and OGT as indicated. C–E, co-immunoprecipitations for OGT using anti-TBP, anti-TFIIF/RAP74, and anti-TFIIH/ERCC3 mAb, along with an anti-OGT immunoprecipitation (rabbit IgG) as indicated. F, co-immunoprecipitations of FCP1 (Santa Cruz Biotechnology) and RNA pol II (BWG16 mAb) and control rabbit and mouse IgG. The Western blot was probed with the anti-OGT antibody AL24. G, nuclear extracts were fractionated over P11 resin and step-eluted in 0.1, 0.3, 0.5, and 1 M KCl (55) followed by SDS-PAGE and Western blotting for OGT. H, aliquots of recombinant His-OGT and His-TBP (1) were assayed by Western blot with an anti-His tag antibody. I, either His-TBP or His-OGT or a mixture of His-TBP and His-OGT was immunoprecipitated with a TBP mAb. Eluates were run on SDS-PAGE followed by a Western blot using an anti-His tag antibody.
between the GTFs and OGT are stably maintained. Consistent with these data is the fact that P11 fractionation of nuclear extract revealed OGT in all four elution steps, each containing different subsets of the GTFs (Fig. 5G). We also assayed for potential interactions with recombinant OGT and TBP. We mixed together His-OGT and His-TBP (Fig. 5H) and immuno-

![Image](image_url)
precipitated with a TBP mAb. Using an anti-His antibody we did not detect any His-OGT, despite recovery of His-TBP (Fig. 5I). From these data we concluded that OGT interacts with several members of the general transcriptional machinery that are known components of the preinitiation complexes that form on promoters.

**OGT shRNA Reduces Transcription and RNA Polymerase II Promoter Occupancy**—The in vitro data points to pol II \(\gamma\) having a specific function in PIC formation, and the in vivo ChIP and ChIP-seq data clearly position pol II \(\gamma\) at the transcription start site of genes. Therefore, we wished to test these ideas and the emerging model (see Fig. 7) by reducing OGT expression in vivo and asking what effects that would have on transcription and ChIP-based detection of factors at promoters. The OGT shRNA was assembled into a lentivirus delivery system that would infect B-cells with high efficiency. Infected cells were selected and individual stable cells lines established. OGT shRNA was induced with the addition of doxycycline, and cells were processed for protein and RNA at 2 days after shRNA induction.

In Fig. 6A we found that at day 2 post-doxycline, OGT mRNA levels were reduced 70% relative to the uninduced cells. We also saw ~50% OGT reduction at the protein level, as assayed by Western blots on whole cell extracts. In contrast, we did not observe any reduction in actin protein levels. We also assayed for decreases in pol II levels by Western blot, comparing uninduced cells to cells at day 2 after shOGT induction. The pol II levels were identical before and after OGT shRNA induction (Fig. 6B). We then assayed several highly expressed B-cell genes and found that OGT shRNA reduced RNA levels 20–60% (Fig. 6C). Lastly, we assayed for the presence of RNA polymerase II, O-GlcNAc, phospho-CTD (serine 5), OGT, and OGA by single locus ChIP (Fig. 6, D–H). We noted significant reductions in O-GlcNAc and OGT, as expected. We also found that both RNA polymerase II and serine 5-phosphorylated RNA polymerase CTD were significantly reduced on all five promoters examined. These data strikingly illustrate the essential requirement of OGT and O-GlcNAc for RNA polymerase II promoter occupancy and transcription.

**DISCUSSION**

**The RNA Polymerase II A/PIC Model**—The current model of PIC formation contains pol II A as the species of pol II capable of forming a PIC. These are based largely on the pioneering experiments of Dahmus and colleagues (7) in the late 1980s and early 1990s, culminating in experiments showing that only pol II A

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**FIGURE 7. A cycle of O-GlcNAcylation on promoter bound RNA polymerase II CTD.** A, the prevailing model of CTD modification of RNA pol II, derived from the work of Dahmus and Reinberg (7–10) and others, is that the unmodified form of pol II (pol IIA) is the initiation-specific form and is the only form of pol II that can form a preinitiation complex. After the initiation of transcription, TFIIH phosphorylates the CTD at serine 5 residues and this becomes the O-form of pol II (pol IIO) (5). B, shown is a schematic illustration of a model that depicts the steps involved in O-GlcNAc-dependent modification of RNA polymerase II. The cycling of O-GlcNAc begins with the arrival of the unmodified pol IIA to the promoter, recruited by other members of the transcriptional machinery (not shown). The pol IIA is converted to pol II \(\gamma\) by the action of the OGT enzyme and UDP-GlcNAc while both proteins are on the promoter. The O-GlcNAc is subsequently removed by the action of the OGA, converting pol II \(\gamma\) back to pol IIA. This removal of O-GlcNAc is necessary for the subsequent initiation/elongation-dependent phosphorylation events by TFIIH and P-TEFb (pol IIO).
forms transcriptionally competent PICs. The exclusion of pol II O is also documented by them and others as well (7, 51). As we mentioned in the introduction, other data imply that pol IIA may not be the only form of pol II associated with the PIC. Kelly et al. (23) show that “unmodified” pol II (pol IIA, purified from calf thymus as described in Ref. 7) contains a population of pol II that is O-GlcNAcylated on the CTD. So the pol II used in experiments described in Ref. 23 to establish the form of pol II that associates with the PIC is likely a mixture of both pol IIA and pol II O. There are several additional caveats to those experiments, including their use of cytoplasmic S100 extracts and not nuclear extracts and the addition of 0.08% Sarkosyl to the transcription reactions (7). We are unaware of any literature in which similar experiments have been done in crude human systems, and data on yeast transcription systems are not relevant because Saccharomyces cerevisiae do not have O-GlcNAc.

Construction of a Model of a pol IIA-containing PIC—The data in Fig. 1 establish that the nuclear extracts are competent, in and of themselves, to convert pol IIA to pol II O. This means that OGT and especially UDP-GlcNAc are present in sufficient quantities to accomplish this conversion. The data in Fig. 2 functionally show that disruption of either OGT or OGA activity abrogates transcription and this transcription defect occurs prior to PIC assembly. The in vitro data in Fig. 3A and the in vivo ChIP data in Fig. 6 show that OGT maps to promoters and is likely part of the PIC. Furthermore, OGT interacts with several GTFs (Fig. 5). Supporting the PIC interpretation further are CTD serine-to-alanine substitutions (Fig. 4), which show that GTFs (Fig. 5). Supporting the PIC interpretation further are ChIP data in Fig. 6 show that OGT maps to promoters and is the direct target of the OGA inhibition. This is supported by the lack of rescue when adding pol II O. This result is not surprising, as the PUGNAc results in the accumulation of pol II O (Fig. 1). There are two other possible interpretations of this experiment, however, which preclude its standing alone. 1) The addition of pol IIA simply sets up a new population of PICs de novo. However, this does not explain why then only pol IIA but not pol II O rescues the PUGNAc block. 2) Why does pol IIA work at all if the OGT and UDP-GlcNAc convert pol IIA to pol II O, which then should be blocked from returning to the A-form by PUGNAc? Note that the likely presence of phosphatases in the experiments of Dahmus and colleagues (7) creates the same caveat to their interpretation. Nevertheless, Fig. 2, E and F, suggest that there is a window of OGT and OGA requirements that perhaps is somehow passed by in the rescue experiment. The limitations of this one experiment, however, are overcome with the in vivo ChIP experiments with pol II O, which support the interpretation of the in vitro data. There pol II O is localized to promoters and the recruitment of pol II O to the promoter is abrogated by the loss of OGT (Fig. 6). These genomic distributions of pol II O and O-GlcNAc are very similar to the results obtained by ChIP-chip analysis of Caenorhabditis elegans (52).

From our in vitro and in vivo functional analysis, we infer that pol II O is a promoter-specific species of pol II. We think that the most likely interpretation of our data is that a cycle of O-GlcNAcylation on the RNA polymerase II CTD occurs on the promoter. This cycle, the “γ-cycle,” creates a form of RNA polymerase II that we term pol II γ. pol II γ is created on the promoter during PIC assembly and is subsequently converted back to an unmodified form before the initiation of transcription (Fig. 7). The complete γ-cycle is necessary for PIC formation, because a block to either step in the cycle, the addition or removal of O-GlcNAc, results in transcription inhibition.

These data open the possibility that pol II is converted from the γ-form immediately to the O-form upon the initiation of transcription, and therefore pol IIA may exist only transiently in the PIC. Secondly, it is now possible that the unmodified form of pol II, pol IIA, exists only as an artifact of its purification, because of the activity of ubiquitous hexosaminidases and the absence of hexosaminidase inhibitors during purification. Indeed, the first purifications of RNA polymerase II from calf thymus contained the pol II γ form (23). Thirdly, these data further support the contention that the content of the PIC is much more complex than suggested in PIC models to date; it is quite likely that many other factors besides OGT and OGA, such as CK2, PARP, and GAPDH, are involved in PIC formation and are present on promoters (28, 53, 54).

The data presented herein argues strongly that there are additional, novel steps in the assembly of PICs on promoters, through either the regulation of the activity of the OGT and OGA or through the temporal regulation of their recruitment to the promoter. It is clear that the regulation of transcription initiation is becoming increasingly complex, far beyond our imagination, and that multiple regulatory steps occur in the proximal promoter region. The continuation of these functional biochemistry studies is mandatory if we are to accurately understand the regulation of transcription in disease and development, as well as the diverse array of promoters and activators that exist in the genome.

Acknowledgments—We thank Kaoru Sakabe for advice, discussions, and early experimental support, Jeff Corden and Shona Murphy for GST-CTD expression vectors, Dirk Eick for critical reading of the manuscript, and D. Reinberg for purified human RNA pol II and antibodies. We also thank Jason Piotrowski for expert technical assistance and Dr. Alfonso Fernandez for experimental assistance.

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