A Mediator-responsive form of metazoan RNA polymerase II

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RNA polymerase II (Pol II), whose 12 subunits are conserved across eukaryotes, is at the heart of the machinery responsible for transcription of mRNA. Although associated general transcription factors impart promoter specificity, responsiveness to gene- and tissue-selective activators additionally depends on the multiprotein Mediator coactivator complex. We have isolated from tissue extracts a distinct and abundant mammalian Pol II subpopulation that contains an additional tightly associated polypeptide, Gdown1. Our results establish that Gdown1-containing Pol II, designated Pol II(G), is selectively dependent on and responsive to Mediator. Thus, in an *in vitro* assay with general transcription factors, Pol II lacking Gdown1 displays unfettered levels of activator-dependent transcription in the presence or absence of Mediator. In contrast, Pol II(G) is dramatically less efficient in responding to activators in the absence of Mediator yet is highly and efficiently responsive to activators in the presence of Mediator. Our results reveal a transcriptional control mechanism in which Mediator-dependent regulation is enforced by means of Gdown1, which likely restricts Pol II function only to be reversed by Mediator.

activator | transcription | repression | negative regulator

The regulated transcription of protein-coding genes, which account for the vast majority of genes in eukaryotic genomes, is a fundamental cellular process. RNA polymerase II (Pol II), one of three related nuclear RNA polymerases (1), is the transcription motor that lies at the core of the transcription machinery for these genes. Although Pol II was first purified and characterized as a complex multisubunit enzyme from mammalian cells (reviewed in ref. 2), the subsequent purification and characterization of yeast Pol II, along with cognate cDNA cloning, allowed rigorous definition of the subunit composition and corresponding genetic analyses (reviewed in refs. 3 and 4). Crystallographic structures of native forms of yeast Pol II confirmed the subunit stoichiometry and content of a 12-subunit enzyme and a related enzyme containing 10 of the 12 subunits (5–7). Furthermore, counterparts to all of the yeast Pol II subunits were found in metazoans (8), leading to the general consensus, in conjunction with biochemical characterization of highly purified metazoan enzymes, that the 12-subunit structure established in yeast is highly conserved from yeast to humans. Although purification of mammalian Pol II did not result in comparably high yields of homogenous Pol II (9–15), studies with essentially pure mammalian Pol II and other transcription factors provided seminal information regarding transcription and its regulation in eukaryotes (1, 16).

As first reported for human Pol II, and despite its structural complexity relative to the transcriptionally competent prokaryotic RNA polymerase, the purified enzyme is incapable of accurately transcribing functional (core) promoters without the assistance of cognate general initiation factors (17, 18). The general transcription initiation factors (GTFs), which together with Pol II form a functional preinitiation complex (PIC), consist of TFIIA, TFIIB, TFIID, TFIIE, TFIIF, and TFIIH and are also phylogenetically conserved (16). Nonetheless, although PIC formation allows for accurate transcription initiation from core promoter elements (e.g., TATA), a robust responsiveness to gene- and tissue-selective DNA-binding transcriptional activators has generally been found to depend, in well defined systems, on the Mediator coactivator complex (19–21).

Mediator activities in yeast and human were discovered on the basis of their function in activator-dependent transcription assays and subsequently identified as multisubunit complexes (19–21). Mediator complexes have been shown to interact both with Pol II (22–27) and, through specific subunits, with transcriptional activators (20, 21). Consistent with these observations, and a role for Mediator as a bridge between activators and the general transcription machinery, Mediator has been shown both to facilitate activator-dependent PIC formation (28, 29) and to act at subsequent steps in transcription (30, 31). Potentially related, the association of Mediator with Pol II results in a structurally altered Mediator conformation (32). Although the isolation of Pol II–Mediator complexes suggested the possibility of joint recruitment, the stepwise recruitment of Mediator followed by Pol II and GTFs has been shown for several promoters (20, 33). Related forms of Mediator that either contain or lack an inhibitory cyclin-dependent kinase 8- and cyclin C-containing module have been described (19–21), although it now appears that the complete complex may be recruited to promoters and that the cyclin-dependent kinase 8–cyclin C module is selectively lost upon transcription initiation (25, 34). Other mechanistic studies have shown that, after initiation, Mediator (along with activator and a subset of GTFs) remains bound at the promoter as a scaffold for successive rounds of transcription reinitiation, suggesting a continuous local effect on transcription (35). Although specific Mediator subunits affect particular developmental or regulatory pathways (19–21), Mediator has been reported to be generally required for transcription of all genes in cells (36) and even for basal transcription in nuclear extracts (22, 37, 38).

The present study has revealed a previously undescribed mechanism for Mediator-dependent activated transcription as a result of an efficient mammalian Pol II purification procedure that revealed two distinct forms of Pol II. Gdown1, a component of a novel Pol II form, acts to repress activation of transcription. Mediator, in turn, relieves Gdown1-induced repression, resulting in high-level transcription.

Results

Pol II Purification. In this report, a mechanism for activator-dependent transcription became apparent as a result of the

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Abbreviations: Pol II, RNA polymerase II; PIC, preinitiation complex; GTF, general transcription initiation factor; CTU, complex transcription unit; rhGdown1, recombinant human Gdown1; TRAP, thyroid hormone receptor-associated protein; PC, positive cofactor; HNF, hepatocyte nuclear factor.

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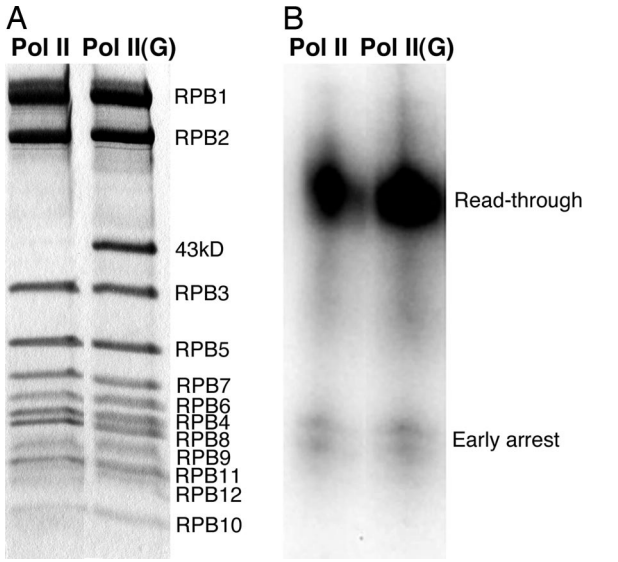

Fig. 1. Polypeptide composition and transcription elongation activities of two forms of Pol II. (*A*) Polypeptide composition after separation on UNO-Q HPLC, resolution on 4–20% SDS/PAGE (NuPAGE), and staining with Coomassie blue. Pol II subunits are numbered according to their yeast Pol II homologues. Identities of subunits RPB3–RPB12 were established by MS analysis. An additional stoichiometric 43-kDa polypeptide is present in Pol II(G). (*B*) Transcription elongation activities in a tailed template assay (39). Both early-arrested and read-through transcripts are indicated. Each reaction contained 1 μg of enzyme, a nonsaturating level that yields an activity in the linear range of the assay (Fig. 6, which is published as supporting information on the PNAS web site).

development of a more efficient purification procedure for mammalian Pol II. This procedure included an affinity purification step with an immobilized monoclonal antibody that recognizes the C-terminal domain of the Pol II RPB1 subunit (14). An unexpected finding from this purification procedure was the existence of a previously undescribed form of Pol II, designated Pol II(G), that was separated from the conventional 12-subunit Pol II by chromatography on an UNO-Q HPLC column (Bio-Rad). Pol II and Pol II(G) share common Pol II subunits but differ in that Pol II(G) contains an additional 43-kDa polypeptide (Fig. 1*A*). Pol II(G) is abundant and represents ≈30% of all purified calf thymus Pol II and 50% of purified pig liver Pol II. Both forms of Pol II are equally active in a nonspecific transcription elongation assay that does not require GTFs for initiation (39) (Fig. 1*B*).

Identification of Gdown1 as a Pol II-Associated Polypeptide. The additional 43-kDa polypeptide in Pol II(G) was positively identified by Edman degradation as Gdown1 (40) and further corroborated by peptide fingerprinting with inferred peptides determined by tandem MS. Edman degradation of peptides from the 43-kDa polypeptide yielded peptide sequence A, AAIAEREEVRGRSELFYPVS, and peptide sequence B, (MYA)QXYNPEGE, where (MYA) represents a pool of possible amino acids and X represents an unidentified amino acid (Fig. 2). Four tandem MS-inferred sequences identifying Gdown1 were MSSLPRGFE, PQKKPHYMEVLE, EIQAKLAAQKLAERLNIKMQSYNPEGE, and RLNIKMQSYNPEGE.

Gdown1 is one of many polypeptide products of the *GRINL1A* complex transcription unit (CTU) (40). Identification of the 43-kDa polypeptide as Gdown1, rather than another GRINL1A CTU product, is evident from a sequence alignment of Edman degradation polypeptides with other GRINL1A products because Gdown1 alone possesses both peptide sequences (Fig. 2*A*).

A

Gdown1	57	KLKAAIAECEEVRRKSELFNPVSLD	337	NIKMRSYNPEFESSG
Gdown2	57	KLKAAIAECEEVGRKSELFNPVSLD	337	LSLAAAAKDTRGSKS
Gdown6	41	----------	180	NIKMRSYNPEGESSG
Gcom1	124	EMRQKIRQLTQELSVSHAQQEYLEN	519	NIKMRSYNPEFESSG
Peptide	A	...AAIAEREEVRGRSELFYPV...	B	..MQXYNPEGE....

B

Human	57	KLKAAIAECEEVRRKSELFNPVSLD	337	NIKMRSYNPEGESSG
Orangutan	57	KLKAAIAECEEVRRKSELCHPVSLD	336	NIKMRSYNPEGESSG
Mouse	57	KLKAAISEREEVRGRSELFHPVSVD	335	NIKMQSYNPEGESSG
Frog	57	KLKAAISEREEVRGRSELFHPVSVD	519	NIKMQSYNPEFESSG
Rat	57	KLKAAISEREEVRGRTELFHPVSVD	519	NIKMQSYNPEFESSG
Bovine	57	KLKAAIAEREEVRGRSELFYPVSLD	519	NIKMQSYNPEFESSR
Peptide	A	...AAIAEREEVRGRSELFYPV...	B	...MQXYNPEGE...

Fig. 2. Comparison of Gdown1 and *GRINL1A* CTU polypeptide sequences. (*A*) Alignment of peptide sequences A and B (determined by Edman degradation) with *GRINL1A* CTU polypeptide sequences sharing common domains. Sequences were predicted from human cDNAs with GenBank accession numbers as follows: Gdown1, AF326773; Gdown2, AK074767 translated from frame 3; Gdown6, AY353061 translated from frame 1; Gcom1, AY207007. (*B*) Alignment of Gdown1 sequences from various species with sequences of polypeptides A and B. Protein sequences were from the Protein Information Resource (PIR) database as follows: Q96JB7, human; Q5REC6, orangutan; Q6P6I6, mouse; Q91XQ4, rat; Q9CXJ7, mouse; Q5U282, frog. The bovine sequence was deduced from partial cDNA sequences from PIR BF042463 and The Institute for Genomic Research sequences TC281745 and TC266399.

Consistent with the above, peptide sequencing verified the origin of Gdown1 as bovine because only calf thymus Gdown1 and not human, orangutan, mouse, frog, or rat contains the tyrosine residue that appears in peptide A (Fig. 2*B*).

Gdown1 as an Authentic Subunit of Pol II(G). Gdown1 appears as tightly associated with Pol II as all other Pol II core subunits. Routine high-salt washes of 500 mM ammonium sulfate used during purification did not dislodge the 43-kDa polypeptide. In *Saccharomyces cerevisiae*, Pol II subunits Rpb4 and Rbp7 are dissociated from the 12-subunit enzyme in the presence of 2 M urea (41). However, no dissociation of Gdown1 from a mixture of Pol II and Pol II(G) was observed on the UNO-Q column in the presence of 2.5 M urea, whereas higher concentrations of urea caused elution of both forms of Pol II. To preclude the possibility that coelution of Pol II and Gdown1 from the UNO-Q column results simply from similar elution properties, recombinant human Gdown1 (rhGdown1) was added to a mixture of bovine Pol II and Pol II(G) and subjected to chromatography on a UNO-Q column. Protein from the single UNO-Q eluted peak was then subjected to size exclusion chromatography in the presence of 2 M urea. Both rhGdown1 and native bovine Gdown1 were observed in the 500-kDa excluded fraction together with Pol II (Fig. 3). It thus appears that Gdown1 tightly binds RNAPII and that a urea-stable Pol II(G) can be reconstituted from rhGdown1 and Pol II.

The chromatographically distinct form of Pol II could be attributed to the associated Gdown1 if the latter were stoichiometric with other Pol II subunits. An assessment of the abundance of Gdown1 relative to subunits RBP3 and RBP5 in Pol II(G) was performed by quantification of seven Coomassie-stained SDS/PAGE gel bands. This analysis included a gel containing a reconstituted Pol II(G) generated by addition of rhGdown1 to a mixture of Pol II and Pol II(G). The average molar ratio of Gdown1 to RBP5 was 1.00, with a standard error of 0.07, and the average molar ratio of RBP3 to RBP5 was 0.91, with a standard error of 0.1. These results indicate a stoichiometric amount of Gdown1 in Pol II(G) and explain the origin of the two forms of Pol II.

From current observations, Gdown1 does not dissociate from Pol II(G) in the presence of urea or high salt, separates with Pol

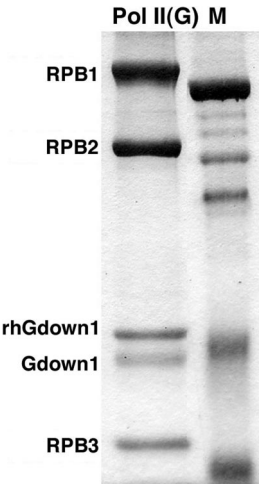

Fig. 3. Tight association of Gdown1 with reconstituted Pol II(G). A mixture of bovine Pol II, bovine Pol II(G), and rhGdown1 was subjected to chromatography on UNO-Q, and the derived, fully reconstituted Pol II(G) was then subjected to size exclusion chromatography in the presence of 2.0 M urea and analyzed by SDS/PAGE and Coomassie staining. M represents marker proteins of 193, 103, 60, 42, and 28 kDa. Rpb1–3, native calf thymus Gdown1, and rhGdown1 are indicated.

II as a distinct form on UNO-Q HPLC, comigrates with Pol II on a sizing column, and is present in stoichiometric amounts in both purified Pol II(G) and reconstituted Pol II–Gdown1 complexes. Evidence in hand thus defines Gdown1 as a component of a previously undescribed form of Pol II.

Mediator-Dependent Function of Poll II(G). Regarding a possible Gdown1 function, strong circumstantial evidence suggested a role for Gdown1 in transcription initiation. First, no functional difference between Pol II and Pol II(G) could be detected in an elongation assay that scores nonspecific (GTF-independent) initiation and subsequent elongation by Pol II (Fig. 1*B*). Second, both previously known Pol II subunits and Gdown1 were found in protein preparations (ostensibly complexes) that were isolated by affinity purification from cells expressing a FLAG-tagged MED26/CRSP70 subunit of the Mediator (27).

This possible association of Gdown1 with a Pol II-containing Mediator complex prompted us to determine whether Gdown1 has a role in Mediator-dependent transcription. We therefore tested Pol II and Pol II(G) preparations in a biochemically defined *in vitro* transcription system that was reconstituted from homogeneous GTFs, positive cofactor (PC)4, thyroid hormone receptor-associated protein (TRAP)/Mediator, or PC2/Mediator and DNA-binding activators (15). As described previously, this system supports efficient activator-dependent transcription from naked DNA templates (15). Under the conditions of the assay, the activator hepatocyte nuclear factor (HNF)-4, a liver- and intestine-enriched orphan nuclear receptor (30), was able to significantly activate Pol II-driven transcription in the absence of Mediator (Fig. 4*A*, compare lanes 6 and 5). The addition of affinity-purified TRAP/Mediator (15) had little, if any, effect on either the basal activity or the already elevated level of HNF-4-dependent activity (Fig. 4*A*, lane 7 versus lane 5 and lane 8 versus lane 6). By contrast, in reactions that contained Pol II(G) but lacked Mediator, activation in response to HNF-4 (Fig. 4*A*, lane 2 versus lane 1) was markedly reduced compared with that seen with Pol II (lane 5 versus lane 6). Intriguingly, the addition of Mediator to the reactions, while again having a marginal effect on basal transcription (Fig. 4*A*, lane 3 versus lane 1), markedly enhanced the level of HNF-4-dependent transcription (lane 4 versus lane 3) to a level that essentially approached that seen with Pol II. In a parallel analysis, the commonly used hybrid activator GAL4-AH effected a strong transcriptional activation with Pol II in the absence of Mediator (Fig. 4*B*, lanes 5–8), whereas a strong transcriptional activation in the presence of Pol II(G) was completely dependent on Mediator (lanes 1–4). Hence, these effects appear to be general rather than activator-specific.

Control experiments (Fig. 4*C*, lanes 1 and 2) in which no Pol II was added established that our system is completely dependent on an ectopic source of Pol II. We also used the PC2 form of Mediator (25), which lacks the potentially repressive MED13/TRAP240-Med12/TRAP230-cyclin-dependent kinase 8/SRB10-CycC/SRB11 subunits (19, 20), to assess whether the interplay between Pol II(G) and the Mediator depends on this module. Given results (Fig. 4*C*, lanes 3–6) that are qualitatively similar to those with the complete TRAP/Mediator complex, we conclude that this effect is a function of the core Mediator. Because PC2/Mediator, by virtue of having undergone more extensive purification [cf. TRAP/Mediator (25)], is devoid of detectable Pol II, these results further

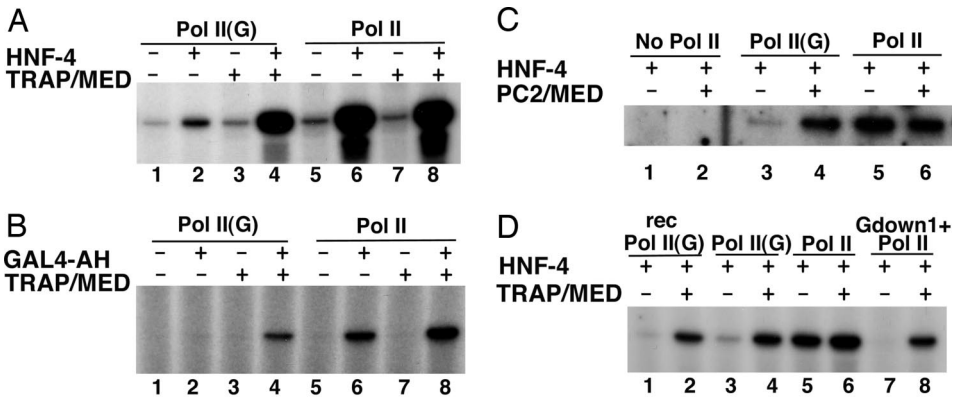

Fig. 4. Mediator is selectively required for activator-dependent transcription by Gdown1-containing Pol II. Transcription was assayed in a completely defined system containing GTFs, PC4, either complete TRAP/Mediator or PC2/Mediator as indicated, either HNF-4 or GAL4-AH and corresponding activator-binding DNA templates as indicated, and either natural [Pol II, Pol II(G)] or reconstituted [rec Pol II(G)] forms of Pol II as indicated. (*A*) HNF-4- and TRAP/Mediator-dependent transcription with Pol II and Pol II(G). (*B*) GAL4-AH- and TRAP/Mediator-dependent transcription with Pol II and Pol II(G). (*C*) HNF-4- and PC2/Mediator-dependent transcription with Pol II and Pol II(G). (*D*) HNF-4- and TRAP/Mediator-dependent transcription with reconstituted Pol II(G). Reconstituted Pol II(G) in *D* was generated either by adding a saturating amount of Gdown1 to a mixture of bovine Pol II and Pol II(G) followed by purification on UNO-Q HPLC (lanes 1 and 2) or by direct addition of Gdown1 to a reaction containing purified Pol II (lanes 7 and 8).

confirm that any observed effects are indeed due to the distinct Pol II forms being tested.

To prove that Gdown1 is responsible for Mediator dependence, we further showed that reconstituted Pol II(G) generated by the addition of recombinant Gdown1 to a mixture of natural Pol II and Pol II(G) is biochemically indistinguishable from natural Pol II(G) (Fig. 4*D*, lanes 1 and 2 versus lanes 3 and 4). Moreover, addition of Gdown1 directly to *in vitro* transcription reactions containing Pol II also altered its activity by conferring Pol II(G)-like characteristics with respect to Mediator responsiveness (Fig. 4*D*, lanes 7 and 8 versus lanes 3 and 4).

Discussion

In this report we demonstrate the existence of a previously undescribed form of mammalian Pol II, designated Pol II(G), that contains Gdown1 as an additional tightly associated peptide. *S. cerevisiae* also contains an alternate form of Pol II, designated Pol IIΔ4/7 (41, 42). However, this form, which is apparently unique to *S. cerevisiae* and prevalent in the log phase of cell growth, represents a different situation in that the enzyme simply lacks two conserved subunits, Rpb4 and Rpb7, that have been linked to stress responses (42). The existence of a Pol II containing an additional subunit(s) has not been previously reported, and the Pol II form described here presents new possibilities for transcriptional regulation.

The high abundance of Pol II(G) relative to Pol II is intriguing because others have reported purification of mammalian Pol II without any apparent identification of Gdown1 as a novel component. However, close inspection of a number of published reports of purified mammalian Pol II preparations reveals, in several cases, copurification of a 41- to 44-kDa polypeptide that is clearly visible on SDS/PAGE gels (11–15). In addition, the fact that some *GRINL1A* CTU transcripts share homology with the NMDA receptor may have led to a misleading appreciation of Gdown1 as NMDA receptor-like and unrelated to transcription. Thus, although Gdown1 appears to have been frequently observed in mammalian Pol II preparations, substoichiometric quantities and insufficient separation of Pol II and Pol II(G), coupled with limited amounts of highly purified enzyme and possibly incorrect informatics, may have prevented its disclosure as a bona fide Pol II-associated polypeptide. In support of the data presented here, MS analysis of affinity-purified human Pol II preparations used in previous studies (15, 25, 43) has confirmed the presence of human Gdown1 (S.M. and R.G.R., unpublished observations). This finding is consistent with observations of significant Mediator-dependent transcription in these studies, whereas the low (but significant) levels of activator-dependent transcription that were observed in the absence of Mediator likely reflect the presence of Gdown1-free Pol II as well (15, 25, 43).

A very striking and significant finding of our results is that homogenous Pol II (lacking any Gdown1) mediates a very robust activator-dependent transcription in the absence of Mediator in a completely purified assay system with GTFs and general coactivator PC4. Moreover, in this system, addition of Mediator has little stimulatory effect. In contrast, in the same system, Pol II(G) is ineffective in mediating activator-dependent transcription unless Mediator is present. Along with the observation that Pol II(G) is fully active in transcriptional elongation after artificial initiation on a tailed template, these results indicate that Gdown1 acts as a direct negative regulator of transcriptional activation and that Mediator relieves this repression and restores activator-dependent transcription to a level equivalent to that observed with Pol II. Our current results contrast with those of a recent report indicating that the yeast Mediator does not act by reversing the action of negative regulators (44). However, the latter results could be consistent with the apparent absence of a Gdown1 counterpart in yeast. On the other hand, our observation of high-level Mediator-independent activated transcription

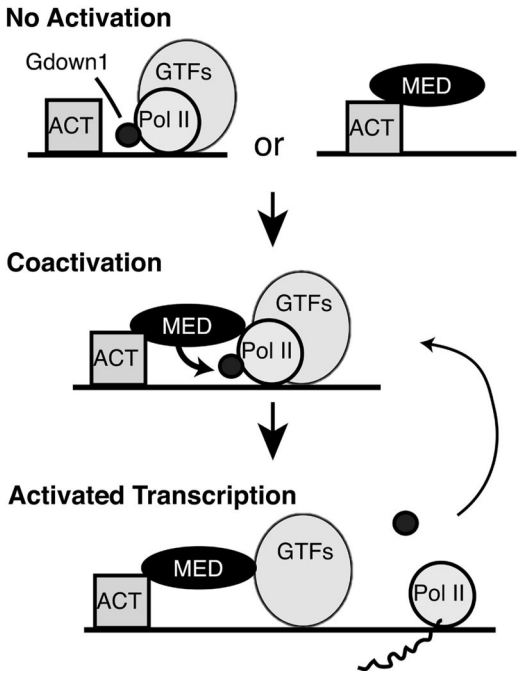

Fig. 5. Model for Mediator-dependent transcription activation by Gdown1-containing Pol II. The model emphasizes formation of an activator–Mediator–Pol II(G)–GTF promoter complex in which formation of a transcriptionally competent Gdown1-free Pol II depends on the presence of the Mediator. The model allows for formation of this complex by divergent pathways, e.g., either by prior formation of an inactive activator-associated PIC followed by Mediator recruitment (*Top Left*) or by formation of an activator–Mediator–promoter complex followed by recruitment of Pol II(G) and GTFs (*Top Right*). Although the fate of Gdown1 after Pol II activation is unknown, it may, as indicated, be released for reassociation with free Pol II and subsequent reentry to the scaffold complex for Mediator-dependent reinitiation.

with Pol II is consistent with a recent report suggesting Mediator-independent activation of some genes in yeast (45). Although the present results do not prove mammalian Mediator-independent transcriptional activation in a more physiological context (e.g., from natural chromatin templates and in the presence of a natural complement of cellular factors), they nonetheless argue strongly for direct activator interactions with other components of the general transcription machinery that could contribute to the overall activation pathway. Many such interactions have been described (4, 16, 46, 47).

The involvement of Gdown1 in the Mediator-dependent activation pathway allows us to propose a model that accounts for the properties of both forms of Pol II (Fig. 5). In this model, Gdown1-containing Pol II is the free form of Pol II involved in PIC formation but not transcription *per se*. Whereas Pol II may be recruited to the PIC, Gdown1 could serve to prevent spontaneous (unregulated) activated transcription. Upon Mediator recruitment, presumably by means of an activator (20, 21), some form of derepression could take place through inactivation and/or removal of Gdown1 from Pol II. This orchestrated series of events would regenerate the 12-subunit Pol II enzyme, which could commence initiation and elongation and perhaps undergo additional modifications, at least in the cellular context, through accretion of various transcriptional elongation complexes and RNA-processing factors (48). Although our data do not shed any light on the eventual fate of Gdown1, a cyclical association–disassociation scenario can reasonably be predicted by analogy with the σ factor cycle described for prokaryotic transcription (49) or for recycling of TFIIF and Pol II in the case of eukaryotes (50). Because Mediator has also been implicated as part of a

reinitiation scaffold that remains once Pol II has moved into the elongation phase (35), it is possible that Mediator facilitates multiple rounds of derepression of newly associating/initiating Pol II(G).

In view of the apparent absence of a Gdown1 ortholog in yeast, it is possible that our results reflect a metazoan-specific regulatory feature. In this regard, it may be relevant that the MED26/CRSP70 subunit, which is greatly enriched in the PC2 (and CRSP) forms of the Mediator (20), represents only one of the few bona fide Mediator components that are not evolutionarily conserved across eukaryotes (51). At the same time, the likely existence of a yeast factor with an analogous function but different (nonconserved) primary structure cannot be ruled out at present.

Our model, which emphasizes tight regulation of the transcription apparatus through a mechanism entailing repression and derepression, is generally consistent with other emerging instances in which potentially active transcription components may be kept in check to preempt dysregulated gene expression. Most prominently, nucleosome-based chromatin structures are generally viewed as repressive to transcription by an otherwise active general transcription machinery, necessitating activator-coupled chromatin remodeling for transcription (1). Other examples include restrictions to TATA-binding protein or TFIID functions through interactions with cofactors such as NC2, Mot1, or the Ccr4–Not complex (46) and negative elongation factor/DRB sensitivity-inducing factor-mediated repression of transcription elongation (48). The existence of multiple levels of tight control at key checkpoints thus appears to be a failsafe device for maintaining normal cell function. As such, derepression of Gdown1-containing Pol II by Mediator may be an effective means to release a transcription "brake," thereby achieving more stringent control at diverse promoters.

Although we have presented what may be a general mechanism for imposing Mediator-dependent transcription, it remains to be determined (*i*) exactly how Gdown1 restricts Pol II function during the formation or function of the PIC, (*ii*) the mechanism by which Mediator relieves this inhibition, and (*iii*) how the chromatin environment of a given gene, as well as other coactivators such as the TAF subunits of TFIID (47), may potentially impinge on the differential usage of Pol II and Pol II(G). Nonetheless, the current study defines a significant aspect of transcriptional regulation through Pol II and the interacting Mediator complex and sets the stage for the further analysis of these important questions.

Materials and Methods

Purification of Pol II. Calf thymus and pig liver were from Pel-Freez Biologicals. Pol II was purified as described (14) with modifications. Generally, 300 g of calf thymus was broken into pieces with a hammer and homogenized in 1 liter of buffer A (50 mM Tris·HCl, pH 7.8/10% glycerol/1 mM EDTA/10 μM $ZnCl_2$) in a 2-liter blender (Waring) for 3 min. The material was centrifuged [GSA rotor (Sorvall) at 11,000 rpm for 20 min], and the supernatant was filtered through Miracloth. Polyethyleneimine was added to the lysate to a final concentration of 0.02%. After stirring for 10 min, the material was centrifuged (GSA rotor at 11,000 rpm for 20 min). The polyethyleneimine pellet was extracted with 400 ml of buffer B [50 mM Tris·HCl, pH 7.8/10% glycerol/1 mM EDTA/10 μM $ZnCl_2$/150 mM $(NH)_2SO_4$]. Lysate was loaded onto a 200-ml MacroPrep High Q Support (Bio-Rad) column, which was washed with three column volumes of buffer B and then eluted with buffer C [50 mM Tris·HCl, pH 7.8/1 mM EDTA/10 μM $ZnCl_2$/500 mM $(NH)_2SO_4$]. The eluate was then subjected to affinity purification on 8WG16-Sepharose, which contains an immobilized monoclonal antibody that recognizes the C-terminal domain of the largest subunit of Pol II, as described by Thompson *et al.* (14). This step was followed by a final step involving UNO-Q column chromatography on a Duo-Flow HPLC System (Bio-Rad). Elution was achieved with a linear gradient of 100–500 mM $(NH)_2SO_4$ in buffer A. Yields were in the range of 6–8 mg of Pol II per kilogram of calf thymus.

Polypeptide Identification. Direct sequencing of the 43-kDa polypeptide was achieved by Edman degradation. The SDS/PAGE-separated 43-kDa band was excised and digested with trypsin after modified in-gel reduction, alkylation, and digestion procedures (52, 53). Protein in-gel slices were denatured and reduced in 6 M guanidine·HCl/10 mM DTT/50 mM NH_4HCO_3 at 55°C for 30 min. This step was followed by alkylation with 50 mM 4-vinylpyridine in 6 M guanidine·HCl/50 mM NH_4HCO_3 at room temperature for 30 min. Gel pieces were washed in 50% acetonitrile/50 mM NH_4HCO_3, dehydrated in acetonitrile, and evaporated to dryness. After digestion with 1.5 μM modified porcine trypsin (Promega) in 50 mM NH_4HCO_3 at 58°C for 30 min, peptides were extracted by sonication in 50% acetonitrile and 5% formic acid and reduced in volume to <25 μl by rotary evaporation. Peptides were fractionated by RP-HPLC on a 0.3×100-mm Clipeus C18 column (The Nest Group), and amino acid sequences of peak fractions were determined by automated Edman degradation with a model 494 Procise sequencer (Applied Biosystems). Confirmation of additional peptides was by standard MS analysis of PolII subunits and the 43-kDa SDS/PAGE bands employing calf thymus Pol II RBP3 as a positive control, performed in the Proteomics Core of the University of Maryland Marlene and Stewart Greenebaum Cancer Center as well as in the Proteomics Resource Center at The Rockefeller University.

Expression of rhGdown1. A human Gdown1 homologue (Lib 969, German Resource Center for Genome Research, RZPD, Berlin) was cloned into pET 100/D-TOPO vector by using the forward and reverse primers 5′-CACCATGTGCTCGCTGCCCCGCGGCTTCGAGC-3′ and 5′-TCAGAATTCATCAGAGGACCAATCGTCATC-3′, respectively, and was expressed in BL21 cells (Invitrogen). The protein induced by isopropyl β-D-thiogalactoside was purified by using a nickel-NTA column as described by the manufacturer (Qiagen). Eluted protein was subjected to HPLC employing a linear gradient from 50–500 mM NaCl on a UNO-Q column (Bio-Rad) in the presence of 50 mM Tris (pH 7.5), 10% glycerol, and 1 mM $CaCl_2$ to maintain protein solubility.

Reconstitution of Pol II(G) with rhGdown1. Purified rhGdown1 was added at a 3-fold molar excess to a natural Pol II preparation containing a mixture of Pol II and Pol II(G). The solution was incubated for 10 min at 4°C and subjected to chromatography on UNO-Q as described for the last stage of the Pol II purification. Protein fractions were precipitated with 50% saturated ammonium sulfate in preparation for a sizing column.

Size Exclusion Chromatography Under Denaturing Conditions. A total of 150 μg of reconstituted Pol II(G) from the above step was resuspended to 50 μl in 2 M urea/50 mM $(NH_4)_2SO_4$/10% glycerol/50 mM Tris·HCl, pH 7.5/1 mM DTT. The solution was incubated for 20 min at 4°C and applied to a Bio-Silect SEC 250–5 sizing column (Bio-Rad), which was preequilibrated in and developed with the same buffer. Fractions were collected, precipitated with trichloroacetic acid, and subjected to SDS/PAGE analysis followed by Coomassie staining.

Transcription Assays. The nonspecific tailed template assay was as described (41). Specific transcription assays with purified components (TFIIA, TFIIB, TFIID, TFIIE, TFIIF, TFIIH, PC4, the indicated Mediators, and the indicated activators and cognate templates) were performed as described (15) and used purified bovine RNA polymerases in place of human Pol II. These included Pol II, Pol II(G), and reconstituted Pol II(G) prepared either as described here or by direct addition of Pol II and Gdown1 to the reaction mixture.

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