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PIC activation through functional interplay between Mediator and TFIIH

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Abstract

The multiprotein Mediator coactivator complex functions in large part by controlling formation and function of the promoter-bound preinitiation complex (PIC), which consists of RNA polymerase II (Pol II) and general transcription factors. However, precisely how Mediator impacts the PIC, especially post-recruitment, has remained unclear. Here, we have studied Mediator effects on basal transcription in an in vitro transcription system reconstituted from purified components. Our results reveal a close functional interplay between Mediator and TFIIH in the early stages of PIC development. We find that under conditions when TFIIH is not normally required for transcription, Mediator actually represses transcription. TFIIH, whose recruitment to the PIC is known to be facilitated by the Mediator, then acts to relieve Mediator-induced repression to generate an active form of the PIC. Gel mobility shift analyses of PICs and characterization of TFIIH preparations carrying mutant XPB translocase subunit further indicate that this relief of repression is achieved through expending energy via ATP hydrolysis, suggesting that it is coupled to TFIIH's established promoter melting activity. Our interpretation of these results is that Mediator functions as an assembly factor that facilitates PIC maturation through its various stages. Whereas the overall effect of the Mediator is to stimulate basal transcription, its initial engagement with the PIC generates a transcriptionally inert PIC intermediate, which necessitates energy expenditure to complete the process.

Graphical Abstract

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Keywords

RNA polymerase II; Mediator coactivator complex; preinitiation complex; promoter melting; triptolide

INTRODUCTION

Transcription initiation by RNA polymerase II (Pol II) is dependent on a set of general transcription factors (GTFs) that enable it to site-specifically form a preinitiation complex (PIC) at promoters of mRNA and miRNA genes (1, 2). In vitro, functional PICs can be generated by the GTFs TFIIB, TFIID, TFIIE, TFIIIF, and TFIIF, which suffice for basal level transcription. These GTFs act in concert to direct and orient Pol II in close proximity to the transcription site (TSS) and coordinate the melting of double-stranded DNA to allow RNA chain synthesis to begin (3). Formation and function of the PIC is rate limiting and it is a major target of transcriptional regulation by gene- and tissue-specific transcription factors (4). Recent studies have suggested a unified general mechanism for initiation by the three eukaryotic RNA polymerases, which are composed of class-specific, as well as shared and paralogous subunits (5). PICs formed by all three polymerases are nucleated by TBP (e.g., as part of TFIID in the case of Pol II) and structural and functional counterparts of all Pol II GTFs except TFIIF have been identified either among the cognate GTFs of Pol I and Pol III or in the non-paralogous subunits of these polymerases. TFIIF is unique among the GTFs in possessing ATP-dependent activities that have been implicated in promoter melting, as well as in possessing a kinase that targets the C-terminal domain of RPB1, the largest Pol II subunit (6).

Pol II is also unique among the three polymerases in being subject to direct regulation by the multisubunit Mediator complex (7, 8). The Mediator was initially identified as a coactivator that interfaces between the regulatory transcription factors and the Pol II machinery to activate transcription (7). Its modular structure, consisting of head, middle, tail and kinase modules, is ideally suited for such a role. Mediator can interact with activators, typically via specific tail subunits (9), and with Pol II, via the head and middle (10–12). However, it is now evident that Mediator does not merely act as a passive conduit for signal transduction between the activators and Pol II. Multiple new functionalities have now been attributed to the Mediator. These include functions at the level of the chromatin template, in enhancer-promoter communication, and negative roles through the reversibly associating Mediator kinase module (7, 8). Importantly, Mediator can also stimulate basal (activator-independent) transcription (13–16). Through interactions with a multitude of cofactors and an ability to

undergo significant conformational changes, Mediator has the potential to function as an integrative node that delivers precisely calibrated outputs to the transcription machinery (7, 8).

Mediator's ability to modulate basal transcription implies a more active role for the complex and suggests that its effects are also exerted after Pol II has been recruited to the promoter, especially since in this context Mediator entry to the PIC is dependent on prior anchoring of Pol II to the template (14). Recent cryo-EM and CX-MS structures of yeast Mediator bound to the PIC has revealed many Mediator-PIC interfaces (11, 17). In addition to the expected contacts with Pol II, the movable jaw of the head module was localized close to the TFIIB B-ribbon domain, in part explaining an earlier observation that excess TFIIB in nuclear extract allows an absolute Mediator requirement for basal transcription in extracts to be bypassed (18, 19). The structures (11, 17) also provide insights into how Mediator can facilitate TFIIF recruitment to the PIC. From its proposed location in the PIC, TFIIF would be well positioned to carry out CTD phosphorylation, and presumably, also DNA melting. Functional and physical interactions between TFIIF and Mediator have previously been described. Mediator has been shown to directly recruit TFIIF to certain yeast loci (20) and to stimulate its CTD kinase activity (21). Conversely, TFIIF can be inactivated by the CDK8 subunit of the Mediator kinase module (22). More recently, it was shown by studies in yeast that TFIIF CTD kinase activity is important, albeit not sufficient, in dissociating Mediator from Pol II as the latter clears the promoters (23, 24).

Here, we report that basal transcription in the presence of Mediator entails close coordination between the Mediator and the ATPase-dependent activities of TFIIF. Using a series of in vitro assays reconstituted from pure preparations of Pol II, general transcription factors and Mediator, we reveal that in the pathway leading to productive transcription, PICs assembled in the presence of the Mediator are initially functionally repressed and that TFIIF's ATPase-dependent activities are critical in reactivation of the PIC. We show further that in the absence of TFIIF activity, the Mediator-induced block is at the earliest stages of nascent RNA formation. These results provide insights into how Mediator can promote PIC maturation through a series of intermediates with distinct functional properties.

RESULTS

Mediator represses transcription initiation in the absence of TFIIF-dependent ATP hydrolysis

Early studies (25) had revealed an energy requirement for transcription in crude assay systems that was subsequently traced to the ATPase-dependent DNA melting activity in the XPB subunit of TFIIF (also known as p89 and ERCC3, and as Ssl2 in yeast) (26). It was further shown that this activity of TFIIF is dispensable when transcription was carried out from supercoiled templates in the presence of Pol II and the other GTFs (27, 28). We now asked how inclusion of the Mediator into PICs reconstituted from highly pure factor preparations might impact energy-dependent steps in early transcription.

Whereas our previous studies have focused on the role of Mediator in activated transcription (29, 30), here we have focused on establishing a simplified assay system to monitor

Mediator effects on basal transcription. For this purpose, we used a G₁₅-STOP template (Fig. 1a) whose core promoter elements were derived from the adenovirus major late (ML) core promoter and in which tandem G-residues were inserted 15 nucleotides downstream of the TSS (30). In the presence of the nucleotides CpA, UTP, and CTP and the chain blocker 3' O-methyl GTP, the G₁₅-STOP template yields both a 16-mer "productive" transcript that is made by Pol II that has escaped (Fig. 1a), as well as a set of abortive and slippage-induced products (31). This template thus allows a more direct readout of events occurring at very early stages of basal transcription. Further, the 16-mer stretch is A-free such that energy dependence of the reaction can be readily controlled by providing or withholding ATP (or dATP, which is interchangeably used in some experiments).

Linearized G₁₅-STOP template was first transcribed in *in vitro* transcription reactions reconstituted with purified and carefully titrated preparations of human Pol II, TBP, TFIIB, TFIIE, TFIIF, and TFIIH (32). As expected, under these conditions, productive transcription of the 16-mer was completely dependent on TFIIH (Fig. 1b, lane 3 vs. lane 1). A circa 4-mer abortive product was, however, observed in the absence of added TFIIH (Fig. 1b, lane 1). While we cannot rule out that trace amounts of TFIIH, which are below MS and Western blotting detection limits are present in our Pol II preparation, especially given that TFIIH is required only in catalytic amounts, we think that it is more likely that the background abortive transcription results from the relatively relaxed conditions of the assay. These include low ionic strength of the reactions and use of a dinucleotide primer, which previously have been observed to give rise to a similar abortive product from the AdML core promoter in the absence of TFIIH (27). More recently, a substantial fraction of closed PICs was found in EM analyses to undergo spontaneous conversion to open complexes (33).

Importantly, the PC2 form of the Mediator stimulated basal transcription several-fold in a TFIIH-dependent fashion in these reactions (Fig. 1b, lane 4 vs. lanes 3 and 2). Thus, this set of factors is sufficient for eliciting Mediator's basal stimulation activity. Note that throughout this study we have used the PC2 form of the Mediator because it lacks the kinase module (14, 29), which by virtue of possessing negative activities would complicate our analysis. Furthermore, to make the functional studies conform better to the binding studies to be described below, as well as recently published structural studies (11, 33–35), we have used TBP in place of TFIID. While TAFs in TFIID may contribute further to Mediator function in some contexts, the results of Fig. 1b indicate that TBP-nucleated PICs can effectively support core Mediator functions in basal transcription.

To understand TFIIH and Mediator interplay at the level of basal transcription in greater depth, we exploited the opportunity afforded by supercoiled templates to bypass a TFIIH requirement. Thus, when transcription reactions were done with supercoiled G₁₅-STOP template, efficient transcription was observed in the absence of TFIIH (Fig. 1c, lane 1), consistent with the well-established ability of PICs formed by only Pol II, TBP, TFIIB, TFIIE, and TFIIF to utilize the energy stored in negatively supercoiled DNA to drive productive transcription (27, 28). Very surprisingly, when Mediator was included in reactions lacking TFIIH, transcription of both the 16-mer and the abortive products was dramatically repressed (Fig. 1c, lane 2 vs. lane 1). This repression was, however, reversed when both TFIIH and dATP (lane 8 vs. lane 1), but not when either of these components

alone (Fig. 1c, lanes 4 and 6), were included in the reactions. We do note that under these highly permissive reaction conditions (including use of supercoiled templates), although TFIIH (together with dATP) was able to modestly stimulate transcription over that seen with the core factors (lane 7 vs. lane 1), further stimulation of basal transcription by the Mediator (Fig. 1c, lane 8 vs. lane 7) was not apparent.

To determine if Mediator repression occurs at relatively early stages of the transcription process, we asked if formation of the first phosphodiester bond is also affected. For this purpose, we monitored a trimeric product (CpApC) that is efficiently generated from supercoiled adenovirus ML templates in the absence of TFIIH and an energy source when only CpA and CTP are supplied (27, 36). As for the 16-mer, synthesis of the trimer was also strongly repressed by Mediator in the absence of TFIIH (Fig. 1d, lane 2 vs. lane 1). The repression was reversed when TFIIH and dATP were also included (Fig. 1d, lane 4). Thus, in the absence of TFIIH, Mediator negatively impacts the PIC at an early step in the formation of a productive transcript.

We also confirmed that in assays using our standard 280 nucleotide-long G-free cassette-containing template that yields longer transcripts, Mediator similarly repressed TFIIH-independent transcription (Fig. 1e). As for the G₁₅-STOP template, this template in supercoiled form and in the presence of the usual NTP mix for G-free transcription, supported basal transcription in the presence of Pol II, TBP, TFIIB, TFIIE, and TFIIF (lane 1). Similarly, TFIIH modestly stimulated this level of transcription (lane 2 vs. lane 1) whereas the Mediator did not have much additional effect (lane 3 vs. lane 2). When, however, ATP in the reaction mix was substituted with ATP γ S, an ATP analog that contains a non-hydrolysable β - γ phosphate bond but can be incorporated into the growing RNA chain, transcription was preferentially inhibited in the presence of Mediator (Fig. 1d, lane 6 vs. lanes 4 and 5). In control reactions, significant rescue of transcription was seen when dATP, which can be hydrolyzed by TFIIH, was added to ATP γ S-containing reactions (Fig. 1e, lane 9 vs. lane 6). The slight inhibition in the case of standard reactions (lane 5 vs. lane 4) can be explained on the basis of an auto-regulatory activity previously ascribed to yeast TFIIH (37). Clearly, however, the fold-inhibition by ATP γ S was significantly greater in the case of reactions containing Mediator, suggesting that the presence of Mediator imposes additional ATP hydrolysis requirements.

These data indicate that under conditions when TFIIH-independent transcription can occur, presence of the Mediator in the PIC is greatly repressive and further that TFIIH in an ATP-hydrolysis dependent fashion can reverse this effect. Results discussed below indicate that this reflects reversal of Mediator-induced repression and not interference by TFIIH of Mediator repression. Further, the data suggest that the Mediator-induced block is very early since production of small RNAs is also repressed.

TFIIH- and ATP-dependent changes in Mediator-containing PICs

To obtain mechanistic insights into how TFIIH-dependent ATP hydrolysis reverses Mediator repression of transcription initiation, we analyzed PICs by EMSA using our previously established conditions for generating Pol II-containing promoter complexes (36). Under our conditions, TBP, TFIIB, TFIIF, and Pol II cooperatively bound to a ML promoter containing

DNA fragment to generate a specific T.B.F.II complex (Fig. 2a, lane 5 vs. lanes 1–4). This complex could further incorporate TFIIE (Fig. 2b, lane 2 vs. lane 1) to yield T.B.F.II.E. However, TFIIH, which based on titrations for our functional studies, was added in sub-stoichiometric amounts, was not apparently stably incorporated, regardless of the absence or presence of TFIIE (Fig. 2b, lanes 3 and 4). When we added saturating amounts of Mediator to the T.B.F.II.E complex (in this case in the presence of TFIIH) it was shifted into a higher order complex that was partially retained at the gel origin (Fig. 2c, lane 3 vs. lane 2). This would be expected for a promoter complex generated as a result of incorporation of the large Pol II-interacting Mediator complex. Control experiments (not shown) established that this gel-origin restricted band was dependent upon Pol II, consistent with Pol II-mediated incorporation of Mediator into basal PICs (14).

Most critically, when the T.B.F.II.E complex was incubated with TFIIH and Mediator in the presence of ATP, no gel origin-restricted signal was detectable (Fig. 2c, lane 6). Instead, the T.B.F.II.E complex was evidently quantitatively converted into a novel, markedly slower migrating complex. Efficient ATP-dependent conversion into this complex only occurred in the presence of both TFIIH and Mediator (Fig. 2c, lane 6 vs. lane 4). Nonetheless, the T.B.F.II.E complex tended to convert into the slower migrating complex even in the absence of Mediator (Fig. 2c, lane 5). These results strongly suggest that the gel origin-retained complex corresponds to the Mediator-containing, but repressed, form of the PIC that became evident from our functional studies (Fig. 1). They further suggest that the slower migrating complex formed as a result of TFIIH-dependent ATP hydrolysis corresponds to an altered (functional) form of the PIC, which while it can form in its absence is stimulated by Mediator. Based on these and other arguments detailed below we have termed this species PIC_a. The origin-retained Mediator-containing complex has been termed PIC_M.

PIC_a is derived from Mediator-bound PIC

In the above assays complex formation was assessed after all components, including ATP/dATP, had been mixed together at initial times. Therefore, the PIC_M and PIC_a EMSA bands potentially could have formed via independent pathways and not necessarily reflect a product-precursor relationship. To distinguish between these possibilities we first made use of our earlier observation that a sub-fraction of the endogenous Mediator in HeLa cells exists in tight association with Pol II as a Pol II-MED (or “holoenzyme”) complex (14). Thus, instead of forming promoter complexes with purified Pol II and ectopically adding Mediator, we used a Pol II-MED preparation in which Pol II is saturated with Mediator. When promoter complexes formed by this preparation in the presence of TBP, TFIIB, TFIIE, TFIIIF, and TFIIH were analyzed by EMSA, a gel origin-retained species that was analogous to the T.B.F.II.E.MED complex seen in Fig. 2c was observed (Fig. 2d, lane 6 vs. lane 5 and lane 2). It can therefore reasonably be concluded that in the absence of ATP hydrolysis Pol II-MED also engages with the GTFs to form a promoter complex. Exposure to dATP, however, yielded a species that migrated exactly as the slower-migrating PIC_a complex that is formed when the T.B.F.II.E complex is converted in the presence of TFIIH, Mediator, and dATP (Fig. 2d, lane 8 vs. lane 4). Note that the relative strengths of the PIC_a signals in the two scenarios reflect the substoichiometric Pol II content of the Pol II-MED preparation (but see also Fig. 6e, lanes 1–4). Importantly, because the Pol II-MED preparation was obtained

through selection via a tagged Mediator subunit (14), the dATP hydrolysis-dependent appearance of this EMSA complex reflects Pol II that has been “delivered” directly by the Mediator.

In a variation of the preceding experiment we next pre-formed the putative T.B.F.II.E.MED promoter complex (PIC_M) by incubating together Pol II-MED and the GTFs (Fig. 2e, lane 1). dATP was then added for varying amounts of time and appearance of PIC_a was monitored by EMSA. PIC_a formation occurred in a time-dependent manner and was essentially complete within minutes (Fig. 2e, lanes 2–4). These results conclusively establish that PIC_a is derived from a Mediator-containing PIC and not formed in a parallel pathway. A very small fraction of the T.B.F.II.E.MED promoter complex (PIC_M) complexes could be converted into PIC_a in the absence of TFIID and an energy source (Fig. 2d, lane 2). As discussed above for abortive product formation, this too may relate to occasional spontaneous opening of the template; role of promoter opening in this process is developed further below.

Altogether, these binding and functional studies strongly suggest a sequence of events in which inactive Mediator-bound T.B.F.II.E complex is converted through TFIID-dependent ATP hydrolysis into a PIC configuration that in EMSA is demonstrably distinct from the initial PIC.

PIC_a is compositionally similar to the PIC and does not contain Mediator

Mobility shifts of EMSA complexes can theoretically result from changes in factor composition, post-translational modifications of proteins, or conformational changes in the nucleoprotein complex. In prior studies (36), we were able to identify the various components of the T.B.F.II complex by using specific antibodies to supershift EMSA complexes. To gauge whether PIC_a contains Mediator, we used antibodies against several Mediator subunits but failed to see any supershifting of the PIC_a band (data not shown). We therefore generated a fluorescent derivative of the Mediator by replacing the C-terminal region of MED1 with cerulean fluorescent protein. Because the N-terminal region of MED1 suffices for interaction with core Mediator (38), purification from nuclear extract of stable cell lines expressing an epitope-tagged fusion protein yielded homogeneous CFP-tagged Mediator preparations (Fig. 3a). In EMSA, this preparation behaved identically to our other Mediator isolates. In the presence of GTFs and Pol II a gel origin-retained band was obtained when the gel was autoradiographed (Fig. 3b, lane 2 vs. lane 1). When dATP was also added, the PIC_a band was efficiently generated (Fig. 3b, lane 4). When the same gel was fluorographed, CFP-tagged Mediator was detected at the gel origin both for reactions done in the absence or presence of dATP (Fig. 3c, lanes 2 and 4). No fluorescence signal was detectable at the expected PIC_a gel location (Fig. 3c, lane 4), suggesting that the PIC_a band is devoid of Mediator. Similar results were obtained with a Pol II-MED preparation derived from CFP-tagged MED1-expressing extracts (Fig. 3d and 3e).

We used mass spectrometry (MS) to more conclusively determine the PIC_a composition. For this purpose we generated the PIC_a EMSA complex on a fluorescent template probe and following fluorography of the wet gel excised the PIC_a band (Fig. 4a, lane 2). An equivalent region from a lane in which we ran reactions that did not contain the DNA probe but

contained all the protein components required for PIC_a formation served as control (lane 1). The results of the MS analyses were displayed as a scatter plot to show the enrichment by label-free quantitation (LFQ, (39)) of proteins in the sample over the control (Fig. 4b, x-axis), an estimate of “absolute” amounts of the proteins by intensity based absolute quantitation (iBAQ; Fig. 4b, y-axis (40)), and number of relevant peptides matched to each of the proteins (Fig. 4b, size of filled circles). The data showed that Pol II subunits were significantly enriched in the PIC_a band relative to the control slice, consistent with complex formation entailing Pol II entry. Unlike what we observed for Pol II, the GTFs TBP, TFIIB, TFIIE, and TFIIIF in the PIC_a band were not apparently significantly enriched relative to the control slice. This only reflects the diffuse presence of these factors throughout each of the two lanes of the EMSA gel and indicates that, unlike for Pol II, their binding to the DNA probe does not necessarily result in these proteins being focused into a sharp band. These GTFs are otherwise known to be integral components of this complex (36). Further, consistent with use of substoichiometric (catalytic) levels of TFIIH in the assay (see also Fig. 2b), its subunits were not strongly detected. Whereas the excised PIC_a-containing gel slice contained many Mediator subunits, like the GTFs, they were not markedly enriched relative to the control gel slice. Moreover, except for MED26 (which was affinity-tagged for Mediator isolation), Mediator subunits were not detected in amounts comparable to that of Pol II. Note the distinct distribution of the Mediator subunit cluster versus the Pol II subunit cluster along the log₂ transformed iBAQ values of the scatter plot in Fig. 4b. Thus, Mediator subunits are not stoichiometric components of the PIC_a band. Taking the above results together, we conclude that, although derived from a Mediator-PIC complex (PIC_M), PIC_a has for the most part lost the Mediator and is an altered form of the PIC.

CTD phosphorylation by TFIIH does not contribute to re-activation of Mediator-repressed PICs

Prior in vitro studies on TFIIH-dependent CTD phosphorylation have suggested that it is conditionally required for productive transcription (41, 42). More recent studies in yeast showed that it contributes to dissociation of Mediator from Pol II (17, 23, 24). We therefore considered if PIC_a is a phosphorylated form of the PIC and used H-8, a pan-kinase inhibitor, to assess how phosphorylation contributes in our reconstituted system. In standard kinase assays, H-8 strongly inhibited in vitro phosphorylation of Pol II RPB1 by TFIIH CDK7 both in the absence or presence of Mediator (Fig. 5a, lane 10 vs. lane 6 and lane 12 vs. lane 8). However, in EMSA, H-8 had little or no effect on the formation of PIC_a (Fig. 5b, lane 12 vs. lane 8). Furthermore, consistent with the homogeneity of our assay system, basal transcription of both productive 16-mer and abortive RNAs from supercoiled G₁₅-STOP template in the absence (Fig. 5c, lane 5 vs. lane 1) or presence (Fig. 5c, lane 7 vs. lane 3) of TFIIH was resistant to H-8. As before, Mediator repressed basal transcription in the absence of TFIIH (Fig. 5c, lane 2 vs. lane 1) but we found that its reversal by TFIIH was not inhibited by H-8 (Fig. 5c, lane 8 vs. lane 4). We conclude from this set of experiments that TFIIH-dependent phosphorylation of the CTD (or other PIC components) contributes minimally, if at all, to the series of events entailing Mediator-repression of the nascent PIC and its reversal by TFIIH to form the PIC_a. Thus, ATPase-dependent activities of TFIIH could be playing a critical role.

PIC_a formation is dependent on catalytic activity through distinct Walker motifs of TFIIH

There is now evidence that XPB functions as a translocase, which by virtue of being tethered to the PIC, can promote initial template melting (3, 26, 37, 43, 44). Like other members of the translocase/helicase superfamily XPB possesses seven Walker motifs (motifs I, Ia, II, III, IV, V, and VI) (45, 46). Individual enzymes in the superfamily have distinct functional roles but share a core catalytic mechanism in which two RecA-like helicase domains (HD) made up of these motifs move relative to each other in an ATP hydrolysis-dependent manner. Previous mutational analyses identified residues K346 in motif I (located in HD1) and T469 and Q638, respectively in motifs III and VI, as critical for XPB function (37).

To both assess if the XPB translocase function is important in the PIC_M-PIC_a transition and to identify the relevant XPB motifs, we generated stable cell lines (Fig. 6a) that inducibly expressed the XPB subunit mutated either in motif I (K346R) or in motifs III and VI (T469A;Q638A) (37). TFIIH was purified from derived extracts and assayed in transcription and EMSA. As expected, both TFIIH^{K346R} (Fig. 6b, lanes 4 and 5 vs. lanes 2 and 3) and TFIIH^{T469A;Q638A} (Fig. 6b, lanes 6 and 7 vs. lanes 2 and 3) were unable to support transcription of the 16-mer RNA from linearized G₁₅-STOP template. Similarly, in the case of transcription from the supercoiled template, unlike wild-type TFIIH (Fig. 6c, lane 2 vs. lane 1), these mutants did not stimulate transcription above the TFIIH-independent levels normally observed from these templates (Fig. 6c, lane 3 vs. lane 1; lane 4 vs. lane 1). Furthermore, these mutants were also unable to reverse Mediator-induced repression (Fig. 6c, lane 7 vs. lane 5; lane 8 vs. lane 5).

In standard EMSA reactions in which PICs were formed by TBP, TFIIB, TFIIE, TFIIF and Pol II, we found that TFIIH^{K346R} was as efficient as wild-type TFIIH in facilitating the Mediator-dependent PIC_M-PIC_a transition (Fig. 6d, lanes 7–9 vs. lanes 4–6) in the presence of dATP. By contrast, TFIIH^{T469A;Q638A} was unable to facilitate this transition above background levels seen when no TFIIH was included in the reactions (Fig. 6d, lanes 10–12 vs. lanes 1–3). Thus, T.B.F.II.E complexes formed in the presence of TFIIH^{T469A;Q638A} bound Mediator efficiently and were retained at the gel origin (Fig. 6d, lanes 11 vs. lane 10) but dATP-induced conversion to PIC_a was compromised (Fig. 6d, lane 12 vs. lane 11).

As in Fig. 2d, we also tested mutant TFIIH preparations in EMSA reactions in which Pol II-MED was used to generate the origin-retained T.B.F.II.E.MED complex (Fig. 6e, lane 1). In the presence of wild-type TFIIH and dATP, PIC_a was efficiently generated in the assay (Fig. 6e, lane 4 vs. lane 2). Whereas TFIIH^{K346R} behaved exactly like wild-type in forming the PIC_a (Fig. 6e, lane 6 vs. lane 4), TFIIH^{T469A;Q638A} yielded only background levels of PIC_a (Fig. 6e, lane 8 vs. lane 2).

These data show that whereas mutations in XPB motif I on the one hand and in motifs III and VI on the other abolish the ability of TFIIH to support transcription from linear templates only mutations in motifs III and VI impact at the level of the PIC_M-PIC_a transition.

The anti-cancer drug triptolide covalently modifies C342 in XPB (47, 48) and inhibits TFIIH ATPase activity without apparently affecting the TFIIH 3' to 5' helicase activity in vitro

assays that monitor release of small oligomers from a partially double-stranded DNA substrate (47). Consistent with published results (47), we first found that synthesis of 16-mer RNA from linearized G₁₅-STOP template, which is completely TFIID-dependent (Fig. 7a, lane 2 vs. lane 1), is strongly inhibited by 10 μ M triptolide (Fig. 7a, lane 4 vs. lane 2). Next we tested the effect of triptolide on the TFIID- and dATP-dependent reversal of Mediator-induced repression of transcription from supercoiled G₁₅-STOP template (Fig. 7b, lane 3 vs. lane 2 and lane 4 vs. lane 3). Under these conditions, triptolide had no effect on TFIID-independent basal transcription in the absence of Mediator (Fig. 7b, lane 5 vs. lane 1). Triptolide did prevent TFIID from further stimulating transcription of the supercoiled template (Fig. 7b, compare lanes 6 and 5 with lanes 2 and 1), presumably because TFIID ATPase activity is important in this secondary effect. Similarly, TFIID-dependent reversal of Mediator repression was significantly reduced, albeit not completely abolished, in the presence of triptolide (Fig. 6b, lane 8 vs. lanes 4 and 7). To more directly assess the effect of triptolide on the PIC_M-PIC_a transition we performed EMSA with Pol II-MED complexes (Fig. 7c). In this experiment we monitored PIC_a in the absence and presence of dATP (Fig. 7c, lane 4 vs. lane 3) and found that triptolide has no effect on its formation (Fig. 7c, lane 8 vs. lane 4).

Collectively, the above results that PIC_a formation is dependent on the DNA melting translocase activity of the XPB subunit of TFIID raised the possibility that this complex is a conformational derivative of the PIC, possibly equivalent to an open promoter complex. Previously, Straney and Crothers (49) were able to readily distinguish closed and open promoter complexes formed by *E. coli* RNA polymerase by EMSA. To directly test this possibility, we compared by EMSA PIC formed on a standard double-stranded DNA probe with an equivalent complex formed on a “bubble” template in which the promoter region (−9 to +2) has been pre-melted (Fig. 7d). As described above, the T.B.II.F.E complex formed on the double-stranded template was converted into a slower-migrating PIC_a by Mediator and TFIID (Fig. 7d, lanes 1–4). However, the T.B.II.F.E complex formed on the bubble template migrated faster than the PIC_a generated on the double-stranded template (Fig. 7d, lane 5 vs. lane 4) and indeed exactly as the corresponding PIC (Fig. 7d, lane 5 vs. lane 1). This result suggests that promoter melting alone cannot account for the difference in the gel mobilities of the T.B.II.F.E and PIC_a bands. We conclude that whereas promoter melting is necessary for the PIC to PIC_a transition, additional structural changes in the PIC also likely occur during the transition.

DISCUSSION

In this paper, we elucidate a Mediator-directed PIC assembly pathway and identify novel intermediates. Our results show that engagement of the Mediator with the PIC, which eventually results in stimulated levels of transcription, initially yields a transcriptionally repressed complex. Subsequent TFIID-dependent ATP hydrolysis, which is coupled to promoter melting, is required to reverse Mediator-induced repression. We propose a multi-step model for Mediator-directed PIC formation, in which PIC assembly proceeds not through passive accretion of its components but through dynamic intermediates.

Mediator as a PIC assembly factor

That we were able to establish a minimal transcription system in which Mediator can stimulate basal transcription *in vitro* allowed us to experimentally distinguish Mediator's contributions to Pol II recruitment from its less well-studied post-recruitment effects. Our analyses revealed that whereas conditions can be found in which transcription is TFIID independent, presence of Mediator in the assay system re-imposes TFIID-dependence, demonstrating that the PIC_M that results from the initial interaction between Mediator and the nascent PIC is a transcriptionally inert variant of the PIC. The requirement for TFIID's XPB translocase activity for reversal of Mediator-induced repression of the PIC and characterization of the electrophoretically identified PIC_a strongly suggested that the latter is a conformational isomer of the PIC whose formation is facilitated by promoter melting (see below). Our results further showed that failure to see Mediator repression when TFIID is present actually reflects interplay between Mediator and TFIID and active neutralization of the Mediator-repressed state (PIC_M) and not simply exclusion of potentially repressive Mediator from the PIC by TFIID. This is evident from the time-dependent conversion of PIC_M to PIC_a from our EMSA assays, which clearly points to a temporal sequence of events in which Mediator effects precede those of TFIID (Fig. 2e). Furthermore, reversal of repression depends not just physically on the TFIID complex but on ATP-triggered XPB translocase activity.

Based on our observations, we propose a revised model for Mediator-directed PIC maturation. As in current models (1, 11, 17, 33–35, 50, 51), PIC formation would begin by TFIID binding to the promoter and formation of a TBP-TFIIB platform. Pol II would next be recruited to the promoter in conjunction with TFIIF. Mediator could enter the PIC either in complex with Pol II or subsequent to Pol II binding to promoter (52), with TFIID joining the PIC last. Different from previous models, our functional and binding studies now allow us to incorporate a number of additional features. One distinguishing feature of the model is that once bound to the Mediator the closed PIC (PIC_M) cannot readily generate a transcription-competent complex even in optimized conditions that do not require TFIID because the Mediator traps a normally transient inactive intermediate state. Additionally, upon Mediator-facilitated incorporation of TFIID into the complex, its ATP hydrolysis-dependent translocase activity both melts promoter DNA and neutralizes Mediator imparted repression. Although it is based on our studies of basal transcription, this pathway should readily extrapolate to the more physiological situation wherein transcription is regulated by activator-recruited Mediator.

Our data indicate that in the transition to PIC_a, Mediator may be stimulating steps that are intrinsic to PIC development. When TFIID is not limiting, low-level PIC_a formation occurs even in the absence of Mediator. Consistent with these observations, we have previously found that under saturating TFIID, Mediator requirements for transcription *in vitro* can be by-passed (19, 32). However, under limiting TFIID, or other stringent conditions, PIC_a formation becomes largely Mediator-dependent. This is best explained through facilitated recruitment of TFIID to the PIC via physical interactions with the Mediator (20) and optimum situation of the XPB subunit on downstream DNA for effecting its translocase function (3, 11, 17, 34). Thus, not only does Mediator association with the PIC impose a

TFIIH translocase dependency on the system it also simultaneously furnishes a means to efficiently elicit the functionality that will subsequently be needed. Recent work in yeast has implicated the CDK7 kinase subunit of TFIIH in Mediator dissociation via phosphorylation of RPB1 CTD (17, 23, 24). However, while this modification may be critical in the *in vivo* context to preclude Mediator re-association over a relatively longer time frame and in the context of other factors that also bind to the CTD, our data clearly indicate that for these earliest transitions that we have focused on here, CTD phosphorylation plays only a peripheral role, if any. The dominant mechanism for countering Mediator's negative side effects on the PIC nucleoprotein complex is through expending energy.

What might be the physical basis for transient repression of PIC_M and its activation by conversion into PIC_a by TFIIH-mediated ATP hydrolysis? Structural studies of Mediator-Pol II complexes reveal Mediator head module interactions near the RPB4/7 stalk, suggesting an allosteric pathway for effecting clamp movements (11, 53). We hypothesize that the PIC_M nucleoprotein complex has distinct structural features, such as a partially open clamp, that might account for why it is unable to initiate transcription. As a corollary, activity would be restored through promoter melting and concomitant clamp closure, potentially also involving additional structural changes in the PIC that go on to yield a stable PIC_a. In this regard, previous measurements of the duration of TFIIH-induced open promoter complexes indicated a very short half-life of 45 sec (54), which contrasts with the relative stability of PIC_a. We note that those studies were done in minimal systems lacking Mediator. Consistent with the proposed assembly factor role of Mediator, it is conceivable that the network of GTF-DNA interactions that stabilize an open complex (33, 34), and presumably in turn promote GTF retention (e.g., of TFIIE (55)), might be greatly favored in the context of the Mediator-directed PIC_a. Absent any evidence for direct Mediator-promoter interactions, the alternative possibility that the effects reported here, especially Mediator-induced repression, are mediated through changes in template topology are less likely. Nonetheless, it is conceivable that Mediator repression might result from indirect effects of the Mediator on the template, as is suggested from recent structural studies of a Mediator-containing PIC (17). Future biochemical and structural studies will allow us to rigorously test our hypothesis and distinguish between the various possibilities.

Implications for TFIIH function

Prior to the realization that the TFIIH XPB translocase activity can account for both promoter melting and escape (43), previous mutational analyses by Gralla and colleagues (37) suggested that the ATPase and apparent helicase activities of XPB in TFIIH might play distinct roles in the PIC. They reported that in their hands the K346R mutation abolished ATPase activity and compromised promoter opening, whereas T469A and Q638A selectively compromised promoter escape. This led them to suggest that the ATPase activity per se is responsible for PIC "remodeling" while the apparent helicase would be responsible for promoter escape (37). Similarly, characterization of triptolide (47), which targets C342 (48), like K346 a residue in the HD1 domain, suggested a selective inhibitory effect on the ATPase and not the apparent helicase function. However, these results pose a paradox because translocase ATPase activity and effector functions must be tightly coupled (45). They also cannot be reconciled with our mutational and pharmacological studies (Fig. 6,

Fig. 7). We believe that the conclusions in the published studies (37, 47) are based on helicase and ATPase assays that may not accurately reflect the full cycle of XPB translocase activity. Our data, on the other hand, allow us to specify for the enzymatic activity of XPB a mechanistic window during which the PIC_M - PIC_a transition occurs. In our hands, the lack of effect of triptolide on the PIC_M - PIC_a transition, but strong inhibition of TFIIF-dependent transcription from linear templates, closely parallels the effects of the XPB K346R mutation. In light of current understanding of how translocase superfamily of enzymes function (45, 56), these data are therefore best explained by a two-stroke mechanism entailing conformational changes in XPB based upon initial ATP binding and its subsequent cleavage. We propose that ATP-dependent PIC_M - PIC_a transition likely occurs in the first step after ATP binds XPB, but before the β - γ phosphate bond is cleaved, and induces relative movement of the RecA like HD domains to trigger at least partial melting of the template (56). In the second step - presumably the one targeted by triptolide and the K346R mutation - β - γ bond cleavage occurs and leads to repositioning of the HD1 and HD2 domains to complete the cycle and effect full melting. Thus, mutations in residues T469 and Q638, which render XPB effector function unresponsive to ATP, likely impact the PIC_M - PIC_a transition because they impart an unfavorable conformation of the HD1 and HD2 domains that interferes with the two-stroke cycle by aberrantly melting DNA and blocking escape, as reported by the Gralla group (37).

Finally, our result that Mediator imposes a TFIIF requirement has implications for the co-evolution of Mediator and TFIIF functions in Pol II regulation. Pol II is the only multisubunit RNA polymerase that requires TFIIF for initial promoter melting (5); a TFIIF requirement for Pol I likely reflects involvement at a post-initiation step (57). The existence of an XPB ortholog in some archaeal species likely also reflects more a role in DNA damage than in transcription (56). Other than TFIIF, all three eukaryotic RNA polymerases display essentially the same level of complexity at the level of their PICs. But only the Pol II system has the additional involvement of the Mediator. As we have described here, the regulatory advantages of the Mediator for the Pol II system come with repressive side effects, which need to be countered by expending energy. Thus, the Pol II general transcription machinery may have expropriated a proto-TFIIF, which originally may have had only DNA damage-related functions, mainly to offset direct Mediator effects on the PIC. As Mediator regulation of Pol II became pervasive, the proto-TFIIF could have evolved into the present day factor that has become an integral part of the Pol II machinery while maintaining dual roles as a GTF and a repair factor.

MATERIALS AND METHODS

Template constructions

The plasmid pA₄X₄ML 53, which contains the 280 bp G-free cassette downstream from the ML core promoter and 4 copies of apolipoprotein AI gene site A (an HNF-4 binding site), has previously been described (58). The “G₁₅-STOP” template was previously derived from pA₄X₄ML 53 by introducing 3 tandem G residues at +15 (30).

Protein expression and purification

Recombinant human GTFs TFIIB, TFIIE, and TFIIF were bacterially expressed and purified as described in detail elsewhere (32). Purification of human Pol II and TFIH from cell lines expressing epitope-tagged subunits has also been described (32). His-tagged TBP was expressed in bacteria and purified over Ni²⁺-NTA agarose and heparin sepharose (36).

To obtain the PC2 form of the human Mediator (29), the VP5-p78 vector (14), which allows expression of FLAG-tagged MED26, was transfected into 293 cells. Stable G418-resistant clones were selected. Nuclear extract from the cells was purified over phosphocellulose P11 and M2-agarose. The isolate derived from the P11 0.5 M fraction contained significant amount of Pol II, and was used in experiments requiring Pol II-Mediator preparations. PC2 was derived from the P11 0.85 M fraction. Both preparations were devoid of CDK8 and other subunits of the kinase module by Western blotting. To obtain the fluorescent (cerulean FP) Mediator derivative, MED(cer-MED1 C), the N-terminus of MED1 (residues 1–630) was fused to full-length cerulean fluorescent protein (59). The fusion protein was expressed in 293 cells via the VP5 vector, as described (32). Nuclear extract was purified over M2-agarose and P11.

Mutant TFIH preparations were also obtained from corresponding cell lines. Using PCR-based methods, the XPB/ERCC3 cDNA (32) was first mutagenized to yield the two derivatives (K346R; T469A/Q638). Note that the numbering system used here is based on Tirode et al (26) and differs slightly from that of the Gralla lab who otherwise targeted these same residues (37). The cDNAs were then subcloned into the pCDNA5/FRT/TO for inducible expression as FLAG-tagged proteins (Thermo Fisher Scientific). After transfection into the flip-in 293 cell line and colony selection (hygromycin-resistant), cells were adapted for growth in suspension cultures. Cells were induced with doxocyclin 24 hours prior to harvesting and nuclear extract preparation. Extracts were purified over DE52, M2-agarose, and P11. As control, wild type XPB was also reengineered for expression via this inducible system.

In vitro transcription assays

Standard in vitro transcription reactions were essentially as described (32). Reactions were reconstituted with Pol II and GTFs with additions of Mediator and TFIH additions, as specified. A typical reaction contained 5 ng TBP, 10 ng TFIIB, 5 ng TFIIE α , 2.5 ng TFIIE β , 25 ng TFIIF, 50 ng Pol II, 100 ng PC2 Mediator, 5 to 20 ng TFIH and 50 ng template DNA. After PIC formation (30 min, 30°C), transcription was initiated by NTP addition followed by incubation for 45 min. For visualization of the 16-mer generated from the G₁₅-STOP template, this NTP mix consisted of 100 μ M CpA, 100 μ M UTP, 25 μ M 3'-O-methyl GTP, and 5 μ M α -³²P-CTP; dATP was typically included at 100 μ M as energy source. Where specified, ATP γ S was also added at 100 μ M; where both dATP and ATP γ S were used, dATP concentration was increased to 200 μ M. Reactions were stopped by heating to 65°C. After cooling on ice, the samples were treated with calf intestinal phosphatase for 20 min and analyzed directly by electrophoresis on 25% PAG containing 7 M urea followed by autoradiography (36). Abortive initiation reactions to monitor the trimeric CpApC product

were performed similarly, except that only 100 μ M CpA and 5 μ M α -³²P-CTP were included in the reactions; dATP was variably included.

Electrophoretic mobility assays

EMSA reactions were performed essentially as previously described (36) except that TFIIE, TFIIH, and Mediator were also included in the analyses. Reaction conditions were essentially the same as for in vitro transcription reactions except that the plasmid template was replaced by an end-labeled DNA probe and the architectural factor PC4 was included to stabilize the EMSA complexes (36). No NTPs were added except for ATP, dATP or ATP γ S, as specified. After an hour-long incubation at 30°C, reactions were loaded on native polyacrylamide gels, which were run in 0.5X TBE buffer. After electrophoresis for 4 hours at 150 V, the gels were dried for autoradiography. For visualization of fluorescent Mediator, the wet gel was directly scanned in a Molecular Dynamics Typhoon 940 scanner (excitation wavelength, 457 nm; emission filter wavelength 520 nm). The bubble template probe was generated by annealing and end-labeling 111-mer oligonucleotides that were complementary except for an 11-base pair mismatch corresponding to the -9 to +2 region of the adenovirus ML promoter, as described (34). A control double-stranded probe was generated by annealing 111-mer oligonucleotides that were fully complementary.

MS analysis of EMSA complexes

For MS-based identification and quantitation of proteins in the PIC_a EMSA band, EMSA reactions were formed on a fluorescently labeled DNA probe. The 115-mer probe was amplified from a plasmid containing the adenovirus ML core promoter using an Alexa Fluor 647-derivatized PCR primer (IDT). The EMSA reactions were performed under standard conditions except that the reactions were scaled up 4-fold and the kinase inhibitor H-8 was included. Control samples lacked template DNA. Following electrophoresis, EMSA complexes were visualized by fluorography of the wet gel (Molecular Dynamics Typhoon 940 scanner; excitation wavelength, 633 nm; emission filter wavelength 670 nm). Regions of interest were excised, cut into pieces and fixed in 45% methanol/10% acetic acid followed by one wash in 30% acetonitrile and shrinking in acetonitrile. Proteins were digested overnight using 10 ng/uL endopeptidase Lys-C (Wako Chemicals) and trypsin (Promega). Extracted peptides were subjected to a C₁₈-Empore based filtering (3M) to remove unwanted gel fragments. Peptides were analyzed by LC-MS/MS (Ultimate 3000 nano-HPLC system coupled to a Q-Exactive Plus mass spectrometer, Thermo Scientific). Peptides were separated on a C₁₈ column (12 cm / 75 μ m, 3 μ m beads, Nikkyo Technologies) at 200 nl/min with a gradient increasing from 1% Buffer B/95% buffer A to 30% buffer B/70% Buffer A in 74 min (buffer A: 0.1% formic acid, buffer B: 0.1% formic acid in acetonitrile) and analyzed in a data dependent (DDA) manner. MS spectra were recorded at 17,500 resolution with m/z 100 as lowest mass. Normalized collision energy was set at 27, with AGC target and maximum injection time being 2e5, and 60 ms, respectively.

DDA data were extracted and queried against UniProt's complete human database (December 2014) concatenated with common contaminants(60) using Proteome Discoverer 1.4 (Thermo Scientific) / MASCOT 2.5.1 (Matrix Science) and MaxQuant (39). N-terminal protein acetylation and oxidation of methionine were allowed as variable modifications and

10 ppm and 20 MDa was used as mass accuracy for precursors and fragment ions respectively. Matched peptides were filtered using 1% False Discovery Rate calculated (FDR) by Percolator (61) and requiring that peptide precursor mass accuracy was better than 5 ppm. MaxQuant results were reported using 1% FDR applied to both protein and peptide matches. Label free quantitation (LFQ) (39) and intensity based absolute quantitation (iBAQ) (40) measures were used to estimate relative and absolute protein amount, respectively.

TFIIH kinase assays

Reactions were assembled under in vitro transcription conditions with factor combinations specified in the figure legend; no template or NTPs were added except for $\gamma^{32}\text{P}$ -ATP. After a one-hour incubation at 30°C, samples were TCA precipitated and analyzed by SDS-PAGE and autoradiography.

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Abbreviations

CX-MS	cross-linking mass spectrometry
CTD	carboxy terminal domain
EM	electron microscopy
GTF	general transcription factor
PIC	preinitiation complex
Pol II	RNA polymerase II
ML	major late
TSS	transcription start site

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HIGHLIGHTS

- Mediator regulates PIC function post-recruitment by poorly understood mechanisms.
- Mediator can trap the PIC in an inactive state.
- Mediator, TFIIH, and ATP cooperatively activate the PIC.

Fig. 1. Mediator represses transcription initiation in the absence of TFIID-dependent ATP hydrolysis

(a) DNA sequence (top) around the initiation site of the G₁₅-STOP template. The expected 16-mer RNA product (italics) generated when CpA is used to prime the reactions. (b) Linearized G₁₅-STOP template was transcribed in in vitro transcription reactions reconstituted with Pol II (II), TBP (T), TFIIB (B), TFIIE (E), and TFIIIF (F); TFIID and PC2 Mediator [MED(PC2)] were variably added as indicated. All reactions contained dATP as an energy source. Production of a 16-mer productive RNA, as well as a cluster of abortive products was monitored. (c) Supercoiled G₁₅-STOP template was transcribed as in panel b; TFIID, PC2 Mediator and dATP were variably added as indicated. (d) Abortive initiation from supercoiled G₁₅-STOP template. Reactions were reconstituted with Pol II, TBP, TFIIB, TFIIE, and TFIIIF, with variable additions of PC2 Mediator and TFIID. Reactions included CpA and α³²P-CTP; dATP was variably added as indicated. Production of a trimeric product (CpApC) was monitored. (e) Supercoiled pA4xML 53 template containing a ca. 280 nucleotide long G-free cassette was subjected to in vitro transcription in reactions reconstituted with Pol II, TBP, TFIIB, TFIIE, and TFIIIF, with variable additions of TFIID and PC2 Mediator. Reactions in lanes 1–3 contained the usual NTP mix for G-free transcription, including ATP. In reactions in lanes 4–6, ATP was replaced with 100 μM ATP_γS; reactions in lanes 7–9 contained 100 μM ATP_γS and 200 μM dATP.

Fig. 2. TFIH- and ATP-dependent changes in Mediator-containing PICs

(a) EMSA complexes on a ML promoter-containing probe were formed in the presence of TBP, TFIIB, TFIIF, and Pol II, as indicated. (b) Higher-order Pol II EMSA complexes formed in the presence of TFIIE and TFIH. (c) PIC_a formation through TFIH-dependent ATP hydrolysis. Pol II EMSA complexes were formed with TBP, TFIIB, TFIIE, TFIIF and Pol II. TFIH and PC2 Mediator were variably added as indicated. Lanes 1–3 show products of standard EMSA reactions; reactions in lanes 4–6 included 100 μ M ATP. (d) EMSA reactions in lanes 1–4 were done essentially as in panel c except that TFIH was included in all reactions; PC2 Mediator and dATP were variably added, as indicated. For reactions in lanes 5–8, Pol II was omitted and a Mediator preparation enriched in Pol II (II-MED) was used as a source of both Pol II and Mediator in reactions in lanes 6 and 8. dATP was added, as indicated. (e) A T.B.F.II.E.MED complex was first generated by pre-incubating the GTFs and II-MED preparation (lane 1). Prior to electrophoresis, dATP was added for variable times, as indicated. Reactions were staggered to allow for simultaneous loading on the gel.

Fig. 3. PIC_a EMSA with a fluorescent Mediator derivative

(a) Silver staining of a purified fluorescent (cerulean FP) Mediator derivative, MED(cer-MED1 ΔC). (b) EMSA analysis of complexes formed in the presence of fluorescent Mediator with factor additions as indicated. (c) Fluorograph of EMSA gel in panel b. (d) As indicated, EMSA reactions were reconstituted with TBP, TFIIB, TFIIE, TFIIIF, and TFIIH but without addition of ectopic Pol II; a Pol II-Mediator preparation derived from extracts expressing the fluorescent (cerulean FP) Mediator derivative, MED(cer-MED1 ΔC) served as source of both Pol II and Mediator in lanes 2 and 4. (e) Fluorograph of EMSA gel in panel d.

Fig. 4. Mass spectrometry of the PIC_a EMSA band

(a) EMSA analysis with a fluorescent DNA probe, with factor additions as indicated. Gel slices excised for MS analyses are highlighted. (b) Comparative MS analysis of polypeptides in the PIC_a EMSA band from panel a. Data are plotted to show both the specific enrichment of the highlighted protein in the PIC_a-containing gel slice versus control and “absolute” protein amount. Log₂ fold-differences based on label-free quantitation (LFQ) are shown on the x-axis; “absolute” amount (derived from intensity-based absolute quantitation (iBAQ) and plotted as log₂ transformed values) are on the y-axis. Relevant proteins have been colored: Pol II subunits (POLR2A–L) in brown; Mediator subunits (MED) in blue; GTFs TFIIB (GTF2B); TFIIE (GTF2E1, GTF2E2); TFIIF (GTF2F1, GTF2F2) in green; TBP in red; and PC4 (SUB1) in black. Reaction contaminants are in grey. The size of the filled circles indicates number of peptides matched per protein (razor plus unique). The complete data set is shown in the inset plot whereas the main panel shows the major protein clusters.

Fig. 5. RPB1 CTD phosphorylation by TFIIH does not contribute to re-activation of Mediator-repressed PICs and formation of PIC_a

(a) Autoradiograph of an RPB1 CTD phosphorylation assay. As indicated, Pol II was incubated with TFIIH and PC2 Mediator in the presence of $\gamma^{32}\text{P}$ -ATP and reactions were processed for SDS-PAGE. H-8 (250 μM) was added to reactions in lanes 9–12. Control reactions in lanes 5–8 were mock-treated with only DMSO, the H-8 solvent. (b) EMSA reactions were set up as in Fig. 2c. Reactions in lanes 9–12 were treated with H-8; reactions in lanes 5–8 were mock-treated with solvent only. All reactions contained dATP. (c) Supercoiled G₁₅-STOP template was transcribed in vitro in reactions reconstituted with Pol II, TBP, TFIIB, TFIIE, TFIIF and variable additions of TFIIH and PC2 Mediator, as indicated. Reactions in lanes 5–8 were treated with H-8; all reactions contained dATP and were adjusted for DMSO.

Fig. 6. PIC_a formation is dependent on distinct Walker motifs in the XPB subunit of TFIIF
 (a) Walker motifs in XPB and residues targeted for mutagenesis. Additionally, the residue covalently targeted by triptolide (C342) is identified. Scheme for obtaining mutant TFIIF preparations is also shown. (b) Characterization of wild-type and mutant TFIIF preparations in basal transcription from linearized G₁₅-STOP template. Reactions were reconstituted with Pol II, TBP, TFIIB, TFIIE, and TFIIF; dATP was used as the energy source. Two normalized concentrations of each preparation were tested. (c) In vitro transcriptions were done as in panel b except that supercoiled G₁₅-STOP template was used. A fixed amount of each of the wild-type and mutant TFIIF preparations was tested in the absence or presence of PC2 Mediator, as indicated. (d, e) EMSA analyses of TFIIF preparations. In panel d, complexes were formed with separately purified Pol II and Mediator, with additions of TFIIF and dATP as indicated; in panel e, PIC_a formation from a Pol II-Mediator complex was monitored as a function of the various TFIIF preparations and dATP.

Fig. 7. Further characterization of the PIC-PIC_a transition

(a) In vitro transcription reactions using linearized G₁₅-STOP template were reconstituted with Pol II, TBP, TFIIB, TFIIE, and TFIIF with dATP as the energy source. TFIH and 10 μ M triptolide (Trip) were added as indicated. (b) In vitro transcription reactions as in panel a, except that supercoiled G₁₅-STOP template was used. TFIH, PC2 Mediator and triptolide were added as indicated. (c) Effect of triptolide on PIC_a formation from a Pol II-Mediator complex was monitored by EMSA. All reactions contained GTFs, including TFIH; Pol II-MED, dATP and triptolide were added as indicated. (d) PIC formation on a bubble template. PICs containing Pol II and the GTFs TBP, TFIIB, TFIIE, and TFIIF were formed on a double-stranded probe (ds; lanes 1–4) or a probe containing a pre-melted bubble (“bub”; lane 5). For reference, PIC_a was generated on the ds probe through combined Mediator and TFIH action (lane 4).