Regulation of transcriptional pausing through the secondary channel of RNA polymerase

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Transcriptional pausing has emerged as an essential mechanism of genetic regulation in both bacteria and eukaryotes, where it serves to coordinate transcription with other cellular processes and to activate or halt gene expression rapidly in response to external stimuli. *Deinococcus radiodurans*, a highly radioresistant and stress-resistant bacterium, encodes three members of the Gre family of transcription factors: GreA and two Gre factor homologs, Gfh1 and Gfh2. Whereas GreA is a universal bacterial factor that stimulates RNA cleavage by RNA polymerase (RNAP), the functions of lineage-specific Gfh proteins remain unknown. Here, we demonstrate that these proteins, which bind within the RNAP secondary channel, strongly enhance site-specific transcriptional pausing and intrinsic termination. Uniquely, the pause-stimulatory activity of Gfh proteins depends on the nature of divalent ions (Mg^{2+} or Mn^{2+}) present in the reaction and is also modulated by the nascent RNA structure and the trigger loop in the RNAP active site. Our data reveal remarkable plasticity of the RNAP active site in response to various regulatory stimuli and highlight functional diversity of transcription factors that bind inside the secondary channel of RNAP.

RNA polymerase | transcriptional pausing | Gfh factors | *Deinococcus radiodurans* | stress response

Cellular RNA polymerases (RNAPs) are complex molecular machines whose activity during transcription is regulated by DNA- and RNA-encoded signals, protein factors, small molecules, and inhibitors. The catalytic cycle of RNAP can be interrupted by pauses of various natures that play important roles in genetic regulation in all organisms, from the classic systems of transcription attenuation and their variations in bacteria (1) to recently discovered widespread promoter-proximal pausing in eukaryotes (2). The pausing serves to activate or repress transcription rapidly at specific genomic sites in response to regulatory stimuli and to coordinate RNA synthesis with other genetic processes (e.g., DNA replication and repair, RNA translation in bacteria) (1, 3–8).

Recent structural and biochemical studies revealed distinct RNAP conformations corresponding to different functional states of the transcription elongation complex (TEC) during nucleotide addition, RNA proofreading, and pausing (9–11). The control of structural transitions between these states likely underlies the function of multiple regulatory factors acting on RNAP. However, the roles of individual RNAP conformations in transcription and the mechanisms of their switching by transcription factors remain only partially understood. During transcription, RNAP holds the DNA template and the RNA transcript within its main channel, whose opening is controlled by the mobile clamp/shelf module and the flap domain of RNAP (9–11). Nucleotide addition and TEC translocation depend on alternating cycles of the folding of the trigger loop (TL) and kinking of the bridge helix (BH) in the RNAP active site (9–11) (Fig. 1*A*). Analysis of the structure of a bacterial paused TEC suggested that the first step in pausing, elemental pause formation, is accompanied by partial clamp opening, TL unfolding, and BH kinking (11). During hairpin-dependent pausing, these changes are reinforced by the nascent RNA hairpin formation under the flap domain of RNAP, resulting in stronger TEC inactivation (12–14).

The secondary channel of RNAP serves as the entry gate for NTP substrates during active transcription and accommodates the RNA 3′-end during TEC backtracking. It also serves as the binding site for regulatory factors, including universal bacterial factor GreA and eukaryotic factor TFIIS, which stimulate RNA cleavage by their cognate RNAPs in backtracked TECs. Gre factors were shown to replace the TL and to chelate catalytic divalent metal ions in the RNAP active site during the cleavage reaction (15–17). Several lineage-specific factors also target the secondary channel, including the DksA protein from Proteobacteria and Gre factor homolog (Gfh) factors from the *Deinococcus/Thermus* phylum of extremophilic bacteria (Fig. 1*B* and Fig. S1). The Gfh1 protein from *Thermus thermophilus* (*Tth*) was shown to inhibit all RNAP activities, including nucleotide addition and RNA cleavage (18–21). Similar to GreA, Gfh1 was proposed to bind metal ions in the RNAP active site by acidic residues from the tip of its N-terminal domain (NTD) (Fig. 1*B*), but to stabilize them in catalytically nonproductive positions (20, 21). In addition, it induces major changes in RNAP conformation (RNAP "ratcheting"), including opening of the clamp/shelf module and kinking of the BH (9, 22). The activity of Gfh1 is regulated by a pH-dependent rotation of its C-terminal domain, resulting in its activation at low pH values (20).

Deinococcus radiodurans (*Dra*) is a highly radioresistant and stress-resistant mesophilic bacterium closely related to *Tth*. It encodes two Gfh factors, Gfh1 and Gfh2, whose cellular functions remain unknown. In this work, we reveal the effects of *Dra* Gfh factors on different steps of transcription and demonstrate that they strongly enhance site-specific transcriptional pausing by *Dra* RNAP. Uniquely, the activity of the Gfh factors is greatly

Significance

Tight and highly controlled transcriptional regulation is pivotal for cell survival under stress conditions. We demonstrate that Gre factor homolog (Gfh) proteins found in the *Deinococcus/Thermus* lineage of extremophilic bacteria affect multiple steps of transcription and may serve to halt transcription at specific genomic sites. These proteins bind within the secondary channel of RNA polymerase (RNAP) and dramatically increase site-specific transcriptional pausing in the presence of manganese ions, likely by stabilizing inactive enzyme conformation. Both Gfh expression and cellular manganese concentration are increased during stress response, thus providing an efficient way for transcription regulation. Similar mechanisms may be operating with other prokaryotic and eukaryotic factors acting through the secondary channel, which emerges as the central regulatory hub in the control of RNAP activities.

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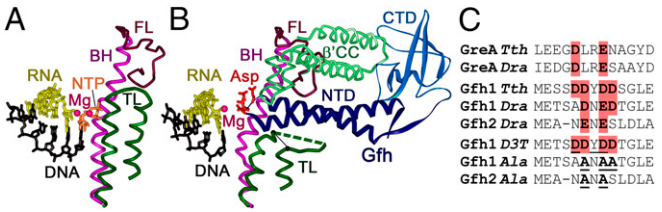

Fig. 1. Interactions of Gfh factors with the RNAP active site. (*A*) Structure of the active TEC of *Tth* RNAP (Protein Data Bank accession number 1IW7) (10). The TL, BH, and F-loop (FL) are shown in green, magenta, and carmine, respectively. (*B*) Structure of *Tth* RNAP–Gfh1 complex (3AOI) (22). The acidic residues in the Gfh NTD tip are red; the β′ coiled-coil element (β′CC) interacting with Gfh C-terminal domain (CTD) at the entry of the secondary channel is light green. The position of the analyzed TL deletion (Δ1254–1272) is shown with a black line. (*C*) Alignment of the active sites of GreA and Gfh factors from *Dra* and *Tth*. Acidic residues in the NTD tip are shown in red; residues substituted in the mutant Gfh factors are boldfaced and underlined. The full GreA and Gfh sequences are shown in Fig. S1.

stimulated by manganese ions, which were previously shown to accumulate in *Dra* cells under stress conditions and to play multiple roles in stress resistance (23–26). We investigate the mechanism of pause stimulation and propose that Gfh factors recognize and stabilize an inactive TEC conformation that is transiently formed at specific pause sites.

Results

Manganese-Dependent Effects of Gfh Factors on Transcription Elongation. We cloned and purified Gfh factors from *Dra* and analyzed their effects on *Dra* RNAP activity at different steps of transcription. All experiments were performed in the presence of either Mg^{2+} or Mn^{2+} ions as RNAP cofactors, because manganese ions were previously shown to play essential roles in stress response in *Dra* (23–26) and to modulate the activity of *Dra* RNAP (27).

We first tested the effects of the *Dra* Gfh factors on transcription initiation. Gfh1 and Gfh2 similarly inhibited abortive synthesis by the *Dra* σ^A RNAP holoenzyme at pH 6.5 and pH 7.9 (approximately three- to fivefold; Fig. S2). No large differences in the inhibition efficiencies were also observed between reactions containing Mg^{2+} or Mn^{2+} ions, except that inhibition by Gfh2 became less efficient in the presence of Mn^{2+} (Fig. S2*B*). Thus, in contrast to *Tth* Gfh1, whose inhibitory activity is greatly stimulated at low pH values (20), *Dra* Gfh factors have only moderate pH-independent effects on transcription initiation.

We next analyzed the effects of *Dra* Gfh factors on the elongation stage of transcription. In the presence of Mg^{2+}, both factors had no effect on the average elongation rate of *Dra* RNAP measured on a 500-bp-long *rpoB*-based template (Fig. 2*B*). In contrast, *Tth* Gfh1 strongly inhibited transcription elongation by *Tth* RNAP (Mg^{2+} reactions; Fig. 2*C*), in agreement with published data (20). Surprisingly, both *Dra* Gfh1 and Gfh2 significantly slowed down the average rate of RNA synthesis in the presence of Mn^{2+} ions, as manifested by the later appearance of full-length transcripts (Fig. 2*B*). Even stronger inhibition was observed for *Tth* Gfh1, resulting in almost complete stalling of transcription by *Tth* RNAP (Mn^{2+} reactions; Fig. 2*C*). Therefore, Mn^{2+} dependence may be a general phenomenon for this family of transcription factors.

Although *Tth* Gfh1 was not functional with *Dra* RNAP, we analyzed a mutant variant of *Dra* Gfh1 with replacement of three amino acid residues in the NTD tip with corresponding residues from *Tth* Gfh1 (Gfh1D3T) (Fig. 1*C*). The Gfh1D3T mutant and wild-type *Dra* Gfh1 had comparable effects on transcription elongation by *Dra* RNAP (Fig. 2*B*), suggesting that the stronger transcription inhibition by *Tth* Gfh1 is not explained by its NTD tip structure but may result from specific properties of *Tth* RNAP and/or differences in RNAP contacts with other Gfh parts (Fig. 1*B* and Fig. S1).

We further analyzed single-nucleotide addition by *Dra* RNAP. The experiments were performed with the minimal nucleic scaffold template that is bound by RNAP in the posttranslocated state and was previously used extensively to characterize catalysis by *Dra* RNAP (27–29) (Fig. S3*A*). The Gfh factors had no effect on the rate of nucleotide incorporation on this template in the presence of Mg^{2+} and only slightly (twofold or less) inhibited it in the presence of Mn^{2+} ions (Fig. S3*A*). Therefore, active posttranslocated TECs are not efficiently targeted by the *Dra* Gfh factors.

***Dra* Gfh Factors Do Not Act as Anticleavage Factors.** Previously, *Tth* Gfh1 was shown to inhibit intrinsic and GreA-dependent RNA cleavage by *Tth* RNAP, suggesting that it may function as an "anti-Gre" factor (18–21). In contrast, *Dra* Gfh factors did not inhibit intrinsic RNA cleavage by *Dra* RNAP in a synthetic TEC with a mismatched A in the RNA 3′-end opposite template G in the presence of Mn^{2+}, and even slightly stimulated it in the presence of Mg^{2+} ions (Fig. S3*B*).

We next tested whether Gfh factors could inhibit GreA-induced RNA cleavage. GreA greatly increased the RNA cleavage rate by *Dra* RNAP in the same TEC (27) (Fig. S3 *C* and *D*); we therefore performed the cleavage reactions not only at pH 7.9 but also at pH 6.5, to decrease the reaction rate and make the measurements more accurate. Titration of GreA, performed at pH 6.5, demonstrated that it binds the TEC with apparent K_ds ($K_{d,app}$s) of 1.3 ± 0.2 μM and 1.7 ± 0.3 μM in the presence of Mg^{2+} and Mn^{2+}, respectively (Fig. S3*C*). When Gfh factors were added in a fivefold molar excess over GreA (5 μM vs. 1 μM) in the presence of Mn^{2+}, they had no effect on GreA-stimulated RNA cleavage, even though the GreA concentration was below its $K_{d,app}$ for the TEC binding (Fig. S3*D*). Thus, Gfh factors cannot efficiently compete with GreA during the cleavage reaction.

Gfh Factors Stimulate Transcriptional Pausing in the Presence of Manganese Ions. The experiments presented above showed that Gfh factors significantly decrease the elongation rate but cannot effectively target transcription complexes involved in RNA synthesis or RNA cleavage. We then proposed that they may recognize a specific state(s) of the TEC formed during transcription elongation. Recent studies suggested that *Tth* Gfh1 stabilizes a ratcheted TEC conformation probably involved in transcriptional pausing and termination (9, 22). We therefore analyzed Gfh effects on transcriptional pausing by RNAP.

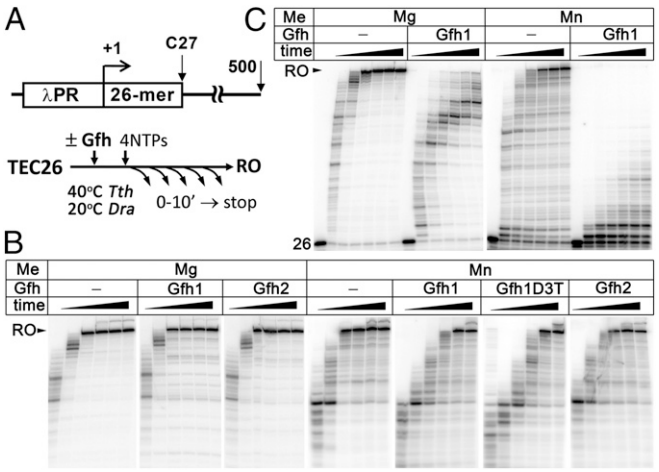

Fig. 2. Effects of Gfh factors on transcription elongation. (*A*) Scheme of the DNA template and outline of the experiment. The λ phage P_R promoter, 26-nt C-less region, and 500-nt run-off RNA (RO) are indicated. The reactions were performed with 10 mM Mg^{2+} or Mn^{2+} at pH 7.9 and stopped after 15 and 30 s and 1, 2, 4, and 10 min. (*B*) Kinetics of full-length RNA synthesis by *Dra* RNAP; Gfh factors were added to 5 μM. (*C*) Effects of *Tth* Gfh1 on RNA synthesis by *Tth* RNAP.

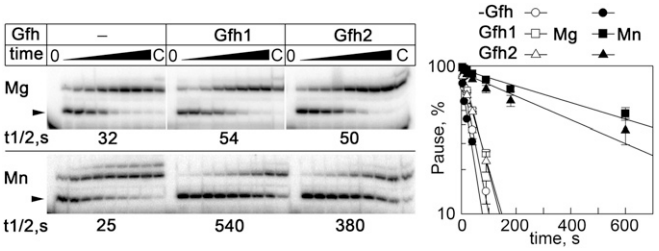

Fig. 3. Effects of Gfh factors on hairpin-dependent pausing by *Dra* RNAP. Analysis of *hisP* pausing was performed at 30 °C in the absence or presence of Gfh factors (5 μM) with 10 mM Mg^{2+} (*Upper*) or 10 mM Mn^{2+} (*Lower*). The paused transcripts are indicated with arrowheads. The pause $t_{1/2}$ times are shown below the gels.

We tested RNAP pausing at a consensus pause site previously identified in genome-wide analyses of transcriptional pausing in *Escherichia coli* (30, 31) and at a hairpin-dependent *hisP* pause site. We used a scaffold-based approach to assemble TECs positioned immediately upstream of the pausing sites (Figs. S4 and S5). For the *hisP* pause site, the RNA hairpin formation was mimicked by the addition of a complementary antisense RNA oligonucleotide (32). Transcription was started by the addition of a limited NTP set, in either the absence or presence of Gfh factors, resulting in the appearance of paused TECs and read-through complexes stalled several nucleotides downstream of the pausing sites (Fig. 3 and Fig. S4*C*).

We found that *Dra* RNAP could recognize both types of the pausing signals in the presence of either Mg^{2+} or Mn^{2+} (Table 1 and Table S1). In particular, the $t_{1/2}$ time of the *hisP* pause for *Dra* RNAP measured in the presence of Mg^{2+} was comparable to *E. coli* RNAP ($t_{1/2}$ = 32 vs. 55 s), and was only slightly decreased in the presence of Mn^{2+} ($t_{1/2}$ = 25 s). Previously, *Tth* RNAP was also shown to respond to the hairpin (11) and consensus (30) pause signals. We confirmed that *Tth* RNAP paused at the *hisP* site, although the pausing was inefficient in the presence of Mn^{2+} ions (Fig. S5*C*). Thus, these signals are universally recognized by RNAPs from different bacterial lineages.

When the reactions were performed with Mg^{2+} ions, *Dra* Gfh factors had essentially no effect on consensus pausing and only weakly stimulated hairpin-dependent pausing by *Dra* RNAP. In contrast, Gfh1 and Gfh2 dramatically stimulated both types of pausing in the presence of Mn^{2+} (Fig. 3, Fig. S4*C*, Table 1, and Table S1). In particular, the pause $t_{1/2}$ times were increased three- to sevenfold for the consensus pause and 15- to 20-fold for the hairpin-dependent pause. In both cases, Gfh factors also significantly increased the pause efficiencies (P_{max}, defined as the maximal pausing at zero time point; details are provided in *SI Materials and Methods*). Similarly, *Tth* Gfh1 stimulated *hisP* pausing by *Tth* RNAP, and the effect was much stronger in the presence of Mn^{2+} ions (twofold vs. >10-fold increase in the pause $t_{1/2}$ in Mg^{2+} and Mn^{2+} reactions; Fig. S5*C*).

Role of the RNA Hairpin in Pause Stimulation by *Dra* Gfh Factors. In subsequent experiments, we focused on the analysis of hairpin-dependent pausing by *Dra* RNAP, which was most strongly affected by Gfh factors. To reveal the role of RNA hairpin in the pause formation, we repeated the experiment without the addition of the antisense RNA oligonucleotide. In this case, the pause $t_{1/2}$ in the absence of Gfh factors was decreased about twofold (14.4 s vs. 25.4 s in Mn^{2+} buffer). Furthermore, the pause-stimulatory effect of the Gfh factors was significantly smaller and was comparable to the consensus pause site (five- to sevenfold increase in the pause $t_{1/2}$; Table 1).

The RNA duplex was shown to affect the conformation of the paused complex significantly, including changes in the clamp domain position and TL folding (12, 13). *Tth* Gfh1 induces similar conformational changes of RNAP (9, 22). We therefore proposed that the RNA duplex formation may stimulate Gfh binding to the TEC. To test this hypothesis, we determined apparent Gfh affinities ($K_{d,app}$s) to the paused TEC by measuring the pausing efficiencies at different Gfh concentrations in the absence or presence of the antisense RNA oligonucleotide (Fig. S6 *A* and *B*). The RNA duplex indeed increased Gfh affinities to the TEC approximately two- to threefold ($K_{d,app}$ = 0.6/0.9 μM and $K_{d,app}$ = 1.2/2.5 μM for Gfh1/Gfh2 with and without the RNA duplex, respectively; Fig. S6*C*). The results suggest that Gfh factors preferably recognize a specific paused TEC conformation that is stabilized by the RNA hairpin.

Gfh Factors Affect the Binding of Metal Ions to the TEC. To reveal whether the inability of Gfh factors to stimulate pausing with Mg^{2+} ions might be explained by their inability to interact with the TEC under these conditions, we compared apparent Gfh affinities with the paused TEC in the presence of Mg^{2+} and Mn^{2+}. Despite the very small effect of Gfh factors on pausing in the presence of magnesium, the $K_{d,app}$ values could be reliably calculated for both Mg^{2+}- and Mn^{2+}-dependent reactions. We found that the affinities of both Gfh factors to the TEC were indeed three- to fourfold lower in the presence of Mg^{2+} ($K_{d,app}$ = 2.3/3.2 μM for Gfh1/Gfh2; Fig. S6 *B* and *C*). However, these differences cannot fully explain the great differences in the pause

Table 1. Effects of Gfh factors on *hisP* pausing

	–Gfh		Gfh1		Gfh2	
Reaction*	$t_{1/2}$, s	P_{max}, %	$t_{1/2}$, s	P_{max}, %	$t_{1/2}$, s	P_{max}, %
WT/Mn^{2+}	25.4 ± 2.5	80.6 ± 1.2	542 ± 86	94.6 ± 0.9	379 ± 90	90.4 ± 1.0
	1		**21.3**		**14.9**	
→ Mg^{2+}	31.6 ± 1.5	90.5 ± 1.4	44.9 ± 2.3	97.3 ± 3.0	48.0 ± 1.2	90.4 ± 3.6
	1		**1.4**		**1.5**	
→ –asRNA	14.4 ± 4.0	74.5 ± 2.4	106 ± 16	92.4 ± 1.0	79.2 ± 7.2	89.2 ± 1.8
	1		**7.4**		**5.5**	
→ Gfh-Ala	—	—	88.9 ± 19.1	79.9 ± 9.2	36.6 ± 5.3	79.1 ± 1.2
			3.5		**1.4**	
→ ΔTL	183 ± 13	77.4 ± 8.4	365 ± 20	81.2 ± 6.4	369 ± 24	79.4 ± 6.2
	1		**2.0**		**2.0**	

*Reaction conditions are shown in comparison to standard reactions performed with WT *Dra* RNAP and Gfh factors in the presence of antisense RNA (asRNA) and 10 mM Mn^{2+}. The arrows indicate components substituted in each reaction. The reactions with the ΔTL RNAP were performed at high NTP concentrations. The numbers in bold indicate the $t_{1/2}$ values in comparison to Gfh-less reactions. P_{max}, projected maximal pausing at zero time point.

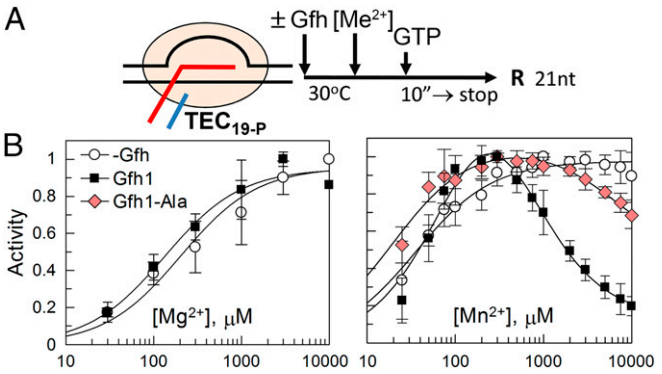

Fig. 4. Effects of Gfh1 on apparent affinities of Me^{2+} ions to RNAP. (*A*) Schematic of the assay. The $TEC_{19\text{-}P}$ complex stalled at the pausing site was assembled from core RNAP and synthetic oligonucleotides, Gfh factors (5 μM) and metal ions were added, and RNA extension to 21 nt was measured after the addition of GTP (2 μM). (*B*) Efficiencies of RNA extension at various Mg^{2+} or Mn^{2+} concentrations in the absence or presence of Gfh1 and Gfh1-Ala. RNA extension at each Me^{2+} concentration was normalized to the maximal extension in the same titration experiment.

stimulation, because Gfh concentrations (5 μM) in all reactions were higher than the $K_{d,app}$ values for Gfh binding.

Previously, Gre and Gfh factors were proposed to chelate divalent metal ions in the RNAP active site by acidic residues in their NTD loops, resulting in activation of RNA cleavage (in the case of Gre factors) or inhibition of catalysis (in the case of *Tth* Gfh1). In particular, both *E. coli* GreA and *Tth* Gfh1 were shown to increase the affinity of metal ions to RNAP (15, 17, 20). To reveal possible effects of *Dra* Gfh factors on the catalytic metal binding, we measured apparent affinities of Mg^{2+} and Mn^{2+} ions to RNAP in the reaction of nucleotide addition in paused TEC in the absence and presence of *Dra* Gfh1. The experiment was performed with the TEC assembled exactly at the *hisP* site; control experiments confirmed that this complex adopts the paused state and is sensitive to the Gfh action (Fig. 4*A* and Fig. S7 *A* and *B*).

In the absence of Gfh factors, titration of Me^{2+} ions resulted in gradual activation of RNA synthesis, with $K_{d,app}$ values of 200 ± 35 μM and 30 ± 12 μM for Mg^{2+} and Mn^{2+}, respectively (Fig. 4*B* and Fig. S7*C*), in agreement with previous measurements for *E. coli* RNAP (33). Because the reaction requires the presence of two catalytic ions in the RNAP active site, these values likely correspond to the binding of the second ion, which is more weakly bound (33). Similar dependence was observed for Mg^{2+} reactions in the presence of Gfh1, which only slightly increased the metal affinity ($K_{d,app}$ = 115 ± 50 μM; Fig. 4*B*, *Left*). In contrast, titration of Mn^{2+} in the presence of Gfh1 resulted in initial activation of RNA synthesis, followed by its inhibition at higher Mn^{2+} concentrations (Fig. 4*B*, *Right* and Fig. S7*C*). We interpret this inhibition as the binding of a third metal ion to the catalytically active TEC, resulting in the decrease in the reaction rate (details are provided in *SI Materials and Methods*). Based on this model, the $K_{d,app}$s of the second and third Mn^{2+} ions derived from the titration curve were 117 ± 11 μM and 520 ± 105 μM, respectively, which lie within the physiological range of manganese concentrations measured in *Dra* cells under stress conditions (23, 24).

To reveal the role of acidic residues at the NTD tip in pause stimulation by Gfh factors, we analyzed mutant variants of Gfh1 and Gfh2 with alanine substitutions of these residues (Gfh-Ala; Fig. 1*C*). The mutant Gfh factors had dramatically impaired ability to stimulate pausing (1.5- to 3.5-fold increase in the *hisP* $t_{1/2}$ in comparison to 15- to 20-fold stimulation for wild-type factors; Table 1). At the same time, these substitutions did not change the Gfh affinity to RNAP ($K_{d,app}$ for Gfh1-Ala of ~0.6 μM in the presence of Mn^{2+}; Fig. S6*C*). Titration of Mn^{2+} ions revealed that the Gfh1-Ala mutant impaired binding of the third Mn^{2+} ion ($K_{d,app}$ > 5,000 μM, at least a 10-fold increase in comparison to wild-type Gfh1), without significantly affecting the binding of the second ion ($K_{d,app}$ = 35 ± 14 μM; Fig. 4*B* and Fig. S7*C*). Thus, the Gfh NTD tip is probably involved in the third ion binding in the vicinity of the RNAP active site, and this ion contributes to transcription inhibition (*Discussion*).

The RNAP TL Is Involved in Stimulation of Transcriptional Pausing by Gfh Factors. The TL in the RNAP active site has previously been implicated in functional interplay with Gre factors, which should replace it to stimulate the RNA cleavage reaction (16, 29, 34). To reveal whether the TL is important for pause stimulation by Gfh factors, we analyzed mutant *Dra* RNAP with a deletion in the TL (Fig. 1*B*). As expected, the deletion significantly increased the *hisP* pause $t_{1/2}$ even when transcription was performed at much higher NTP concentrations in the presence of Mn^{2+}, likely because it decreased the rate of RNA synthesis. However, the Gfh factors were almost unable to stimulate pausing by the mutant RNAP (only a twofold increase in the pause $t_{1/2}$; Fig. S5*D* and Table 1). Titration of Gfh1 revealed that the TL deletion significantly decreased its affinity to the mutant RNAP ($K_{d,app}$ was increased ~14-fold; Fig. S6*C*). Thus, the TL is likely involved in Gfh binding to RNAP and in transcription inhibition.

Gfh Factors Stimulate Intrinsic Transcription Termination. The strong stimulation of hairpin-dependent pausing by Gfh factors prompted us to investigate their effects on intrinsic termination, which also depends on the RNA hairpin formation. Both Gfh1 and Gfh2 only slightly increased termination efficiency on a model λ phage tR2 terminator in the presence of Mg^{2+} ions (Fig. 5). Interestingly, the termination efficiency by *Dra* RNAP was significantly decreased when transcription was performed with Mn^{2+} ions. The addition of Gfh factors restored termination in the presence of Mn^{2+} (Fig. 5*B*). Therefore, Gfh1 and Gfh2 can act as termination factors under these conditions.

Finally, we tested the effects of Gfh factors on Rho-dependent termination. Because no Rho-dependent terminators from *Dra* have been described to date, we analyzed termination on a genomic *Dra* fragment corresponding to the *E. coli trpt'* terminator located after the α-synthase gene in the conserved tryptophan operon. The addition of *Dra* Rho to the transcription reactions resulted in the appearance of terminated RNA products in the expected region (Fig. S8). Gfh1 and Gfh2 did not affect Rho-dependent termination. Thus, the Gfh factors do not generally make RNAP more prone to transcription termination, but act on only a subset of termination signals.

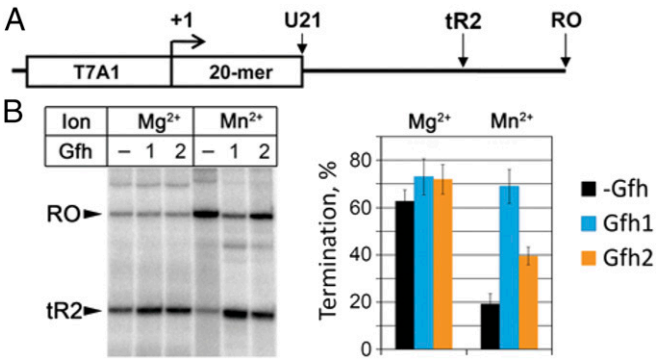

Fig. 5. Effects of Gfh factors on intrinsic transcription termination by *Dra* RNAP. (*A*) Scheme of the template. Positions of the T7A1 promoter, 20-nt U-less region, λ phage tR2 terminator, and run-off RNA are shown. (*B*) Analysis of transcription termination was performed in the presence of 10 mM Mg^{2+} or Mn^{2+} ions at 37 °C. Termination efficiencies are shown as the percentage of the terminated (tR2) transcripts relative to the sum of tR2 and RO RNAs.

Discussion

Gfh factors found in extremophilic bacteria from the *Deinococcus/Thermus* phylum belong to the notable family of transcription factors that bind within the secondary channel of RNAP and directly modulate its catalytic activity, but their functional role in transcription remains largely unknown. We demonstrate that the Gfh1 and Gfh2 proteins from the radioresistant bacterium *Dra* preferentially target transcription complexes involved in pausing and termination, and are able to halt RNAP at specific genomic sites. In particular, both Gfh1 and Gfh2 strongly enhance recognition of two common types of the pausing signals found in bacteria, consensus and hairpin-dependent pauses, by *Dra* RNAP. They also stimulate intrinsic termination, which likely proceeds through the same structural intermediate(s) as hairpin-dependent pausing (9, 35, 36). Previously, the pause RNA hairpin was shown to induce long-range conformational changes of the TEC, including opening of the clamp domain and inhibition of the TL folding (12, 13). These changes likely stabilize the initial elemental paused state, which is common for various pause types (11), and improve Gfh binding in the secondary RNAP channel, thus enhancing their effects on pausing. Because the Gfh factors inefficiently inhibit ΔTL *Dra* RNAP (whereas the TL deletion by itself stimulates pausing, likely by decreasing the rate of nucleotide incorporation), the unfolded TL likely contributes to Gfh binding, either directly or by changing the conformation of the secondary RNAP channel, and may be required for pause-associated TEC rearrangements. In the paused TECs, the secondary channel and the active center of RNAP are partially occluded by Gfh, the TL is unfolded, and the binding site of the incoming nucleotide is occupied by the kinked BH, thus inhibiting nucleotide addition (11, 22).

Notably, both consensus and *hisP* pauses that are stimulated by Gfh were shown to be resistant to the action of Gre factors (30, 37). GreA is also unable to target active TECs involved in RNA synthesis (9, 16). Conversely, the *Dra* Gfh proteins have only minor or no effects on intrinsic and GreA-stimulated RNA cleavage in mismatched TECs, although both types of factors have comparable affinities to their target TECs. Thus, the two classes of factors likely recognize distinct structural states of the TEC. Recent structural studies revealed different degrees of RNAP ratcheting (clamp opening and BH kinking) in GreA- and Gfh1-bound *Tth* TECs (9, 11, 22), probably explaining the different TEC specificities that we observed for the *Dra* GreA and Gfh factors. Although *Tth* Gfh1 can induce conversion of active TECs into the inactive conformation (9, 20), the *Dra* Gfh factors have likely evolved specifically to target paused TECs that are transiently formed during transcription, resulting in weaker average effects on transcription elongation in comparison to *Tth* Gfh1.

E. coli Gre factors and DksA were also shown to target different types of the TEC, thus avoiding direct competition between these factors during transcription elongation (38). Although the exact TEC state targeted by DksA remains to be identified, both DksA and Gre ultimately suppress formation of backtracked complexes by *E. coli* RNAP and decrease the transcription/replication conflicts (5, 39). In addition, DksA was proposed to decrease nucleotide misincorporation and associated transcriptional pausing (40, 41). The Gfh factors might also prevent TEC backtracking at the sites of pausing, by stabilizing inactive TECs that could be further disassembled by cellular machineries involved in DNA replication and repair (3).

The *Dra* Gfh factors depend on manganese ions for their effects on transcriptional pausing and intrinsic termination, but not on transcription initiation, suggesting that the mechanisms of inhibition might be different for different steps of transcription. Our experiments revealed that the Gfh factors likely promote binding of an additional (third) Mn^{2+} ion to RNAP, which may help to stabilize the paused TEC conformation. Alanine substitutions of acidic residues in the Gfh1 NTD interfered with its action and impaired the third ion affinity, suggesting that the NTD tip contributes to the Mn^{2+} binding at or near the RNAP active site, either directly or through interactions with other elements in the active site (20, 22) (Fig. 1*B*). Mn^{2+} ions, by themselves, may also affect the active site structure and the TL conformation (27, 42, 43), thus making RNAP more sensitive to the Gfh action and increasing its affinity to Gfh.

Manganese ions were shown to reach millimolar concentrations in *Dra* under stress conditions (23, 24). Importantly, manganese accumulation was also observed during stress response in other bacterial phyla as well as in eukaryotic organisms (44). Although the data on intracellular distribution of Mn^{2+} ions in *Dra* are controversial (26, 45–47), several reports suggest that a significant fraction of Mn^{2+} ions is bound to proteins (46, 48). Accordingly, Mn^{2+} ions were shown to activate several stress-induced enzymes in *Dra*, including DNA polymerases (49, 50), Mn^{2+}-dependent superoxide dismutase (45, 48), and the PprI protease responsible for activation of dozens of stress-related genes through the cleavage of DdrO repressor (51). Our data suggest that RNAP is another likely target for manganese-dependent regulation in *Dra*. In support of this hypothesis, the expression of Gfh1 (DR1970) and GreA (DR1162) in *Dra* is strongly induced after irradiation, although Gfh2 (DR2375) is expressed constitutively (52, 53). Notably, the effects of Gfh1 on transcriptional pausing are stronger than the effects of Gfh2, likely resulting in large overall changes in transcription. Gfh-dependent and Mn-dependent stimulation of transcriptional pausing and termination may therefore serve as a specific mechanism of genetic regulation during stress response and may help to coordinate transcription with other genetic processes.

Although little is known about the role of manganese in stress resistance in *Thermus*, there is evidence that cellular Mn^{2+} concentrations are sufficiently high in *Tth*, and several Mn^{2+}-dependent enzymes have been implicated in the oxidative stress response in this bacterium (54–56). We found that although *Tth* Gfh1 is functional in the presence of magnesium ions, its pause-stimulatory activity is also greatly enhanced by manganese. In line with this finding, *Tth* Gfh1 was shown to strongly inhibit exonucleolytic RNA cleavage by *Tth* RNAP in the presence of Mn^{2+} ions (21), and to have different effects on transcription initiation depending on divalent metal ions included in the reaction (20). We therefore propose that the activity of Gfh factors from various bacteria may be similarly modulated by the type of metal ions present in the transcription reaction.

Recently, a DksA2 paralog of DksA in *Pseudomonas aeruginosa*, which lacks the Zn-finger motif required for the function of the canonical factor, was shown to be induced under Zn-limiting conditions and to regulate transcription of virulence genes through binding to RNAP (57). Altogether, these observations extend the concept of factor-dependent exchange of RNAP activities in response to various regulatory stimuli (16, 21, 34), and suggest that various secondary channel regulators may play a general role in metal homeostasis and, more broadly, in adaptation to complex ecological niches.

At least some details of the pausing and termination pathways appear to be similar in bacterial and eukaryotic RNAPs. In particular, mammalian RNAP II was shown to respond to the bacterial consensus pause signal (30) and yeast RNAP III was suggested to recognize RNA hairpins during termination (58). Furthermore, an open, ratcheted RNAP conformation was observed in eukaryotic RNAP I (59, 60) and in archaeal RNAP (61). Thus, similar mechanisms of transcriptional regulation by secondary channel factors that affect RNAP conformation may function in other domains of life.

Materials and Methods

The detailed experimental procedures are described in *SI Materials and Methods*. RNAP pausing was analyzed using synthetic nucleic scaffold templates at 30 °C in in the presence of 10 mM $MgCl_2$ or $MnCl_2$ and 5 μM Gfh factors, unless otherwise indicated.

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