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In Vitro Transcriptional Analysis of TyrR-Mediated Activation of the *mtr* and *tyrP+3* Promoters of *Escherichia coli*

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In order to understand the mechanism by which the TyrR protein activates transcription from the *mtr* and *tyrP+3* promoters, we have carried out in vitro transcription experiments with supercoiled DNA templates. We have shown that addition of the histone-like protein HU or integration host factor (IHF) greatly inhibited the transcription from the *mtr* and *tyrP+3* promoters. In the presence of phenylalanine, the wild-type TyrR protein, but not a mutant TyrR protein (activation negative), was able to relieve the HU- or IHF-mediated inhibition of transcription. In contrast, the alleviation of the HU- or IHF-mediated transcription inhibition by the wild-type TyrR protein did not occur when a mutant RNA polymerase with a C-terminally truncated α subunit was used to carry out the transcription reaction.

In *Escherichia coli*, the expression of both the *mtr* and *tyrP* genes, which code for the tryptophan- and the tyrosine-specific permeases, respectively, can be activated at the level of transcription by the TyrR protein. In the case of *mtr*, the activation by the TyrR protein is mediated either by tyrosine or by phenylalanine. The extent of activation varies from 3- to 10-fold, depending on the genetic background of the strain used (14, 26), and increasing the gene dosage of $tyrR^+$ by introducing a multicopy plasmid carrying the $tyrR^+$ gene into the cell results in the increased activation of the *mtr* gene (26). In a *trpR* mutant background, tryptophan can also mediate transcription activation of the *mtr* gene by TyrR, but the extent of activation is slighter than that mediated by phenylalanine or tyrosine (26). In the case of *tyrP*, its expression can be activated 2- to 3-fold by TyrR in the presence of phenylalanine or repressed 20-fold by TyrR in the presence of tyrosine (2, 20).

In vivo and in vitro studies have identified TyrR binding sites (TyrR boxes) in the upstream regions of both the *mtr* and *tyrP* genes (2, 20, 25, 26). In *mtr*, the TyrR box (strong box), which plays a major role in TyrR-mediated activation, is centered at −76.5 or −77.5. In *tyrP*, the TyrR box (strong box), which is responsible for TyrR-mediated activation, is centered at −64.5. The strong TyrR box of *tyrP* is not positioned ideally for activation, because moving it 3 to 4 or 13 to 14 bases further upstream increases the extent of activation to 10-fold (1). With the strong box in these positions, both phenylalanine and tyrosine can mediate the activation by TyrR (1).

The TyrR polypeptide contains 513 amino acid residues which constitute three structural domains (4, 6, 35). Mutational studies have identified a number of amino acid residues in the N-terminal domain of TyrR which play an essential role in activation (5, 33, 34). Lawley et al. have studied the TyrR-mediated activation of the wild-type *tyrP* promoter by using an in vitro transcription system and have provided experimental evidence indicating that TyrR protein activates the transcription of *tyrP* through direct contact with the C-terminal portion of the α subunit of RNA polymerase (23). On the basis of these results, the TyrR protein has been proposed to be a class I transcriptional activator.

Previously, in vitro transcription experiments with a linear DNA template containing either the *mtr* promoter or a variant *tyrP* promoter, *tyrP+3* (three bases were inserted between the strong TyrR box and the *tyrP* promoter), have also been performed in our laboratory. However, in both cases, we were unable to demonstrate the level of TyrR-mediated activation which correlates to that observed in vivo (results not shown). In this study, we readdress this issue by using a modified in vitro system, and we show that the presence of additional cellular factors is necessary for the TyrR protein to activate transcription from the *mtr* or *tyrP+3* promoter.

Effect of DNA supercoiling and HU on transcription and activation of the *mtr* and *tyrP+3 promoters.** In the present work, we have used supercoiled DNA templates, which are closer to the topological state of DNA in vivo, to investigate the nature of TyrR-mediated activation of the *mtr* and *tyrP+3* promoters. The supercoiled templates were derivatives of plasmid pDD3, which was specifically designed for in vitro transcription (29). The pDD3 plasmid contains two T_1 transcription terminators from the *Escherichia coli rrnB* ribosomal operon, which are situated 150 bp on either side of a unique *Bam*HI site. We inserted each of the two *Bam*HI fragments which contained the promoters of *mtr* (280 bp) and *tyrP+3** (327 bp) into the *Bam*HI site of pDD3 to form plasmids pDD3-mtr and pDD3-tyrP+3*. The *tyrP+3** template carried, in addition to the three-base insertion between the strong TyrR boxes and the *tyrP* promoter, a base substitution which changed A to C at position −75 from the transcriptional start site of *tyrP*. This mutation alters the invariant T of the −10 region of promoter X, which transcribes from the opposite strand to the *tyrP* promoter (23) and minimizes the strong transcription from this opposite promoter in vitro (23).

Initially, we performed in vitro single-round transcription experiments with wild-type RNA polymerase and the supercoiled template pDD3-mtr or pDD3-tyrP+3*. The transcription reactions were carried out in the presence or absence of the purified TyrR protein with or without phenylalanine. The products from the reactions were analyzed by electrophoresis on denaturing 6% polyacrylamide gels. The expected lengths of the in vitro transcripts are 236 bp from the *mtr* promoter and 252 bp from the *tyrP+3** promoter. As shown in Fig. 1, each

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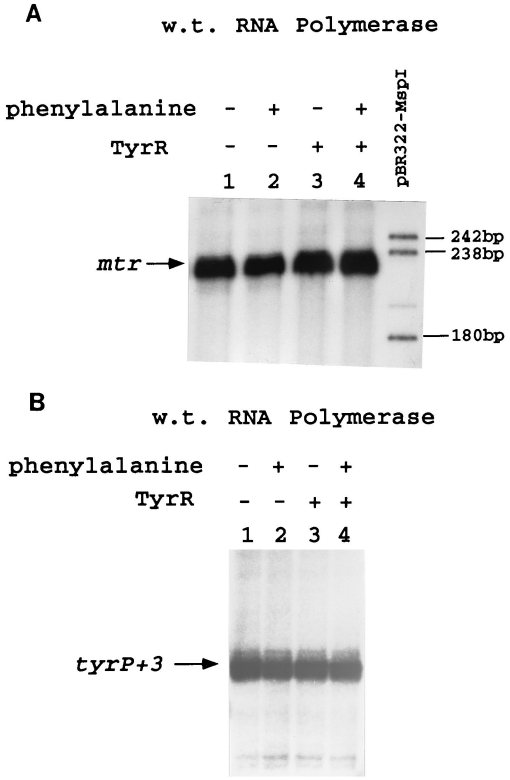

FIG. 1. (A) In vitro transcription from the *mtr* promoter. Single-round in vitro transcription was performed as described by Igarashi and Ishihama (18). The reaction mixtures contained 0.5 pmol of wild-type (w.t.) RNA polymerase and 0.015 pmol of plasmid pDD-mtr. When added, the final concentrations of the TyrR protein and phenylalanine were 200 nM and 1 mM, respectively. (B) In vitro transcription from the *tyrP+3* promoter. A total of 0.015 pmol of DNA template (pDD3-tyrP+3*) was used for each transcription reaction mixture. Single-round in vitro transcription was performed under the conditions described in the legend to Fig. 1A.

template produced a single abundant RNA product, the size of which corresponded to that of the predicted transcript from the relevant *mtr* or *tyrP+3** promoter. The levels of the mRNA products, from both the *mtr* and *tyrP+3** promoters, synthesized from the supercoiled templates are at least 20- to 30-fold higher than those synthesized from linear DNA templates (results not shown) under the same assay conditions. However, no significant stimulation of transcription from either the *mtr* or the *tyrP+3** promoter by TyrR and phenylalanine was seen. We reasoned that the lack of TyrR-mediated activation in the in vitro assay might be due to the presence of saturating levels of RNA polymerase in the reaction mixtures or the absence of an essential cellular component(s) in the system. To test the first possibility, we carried out transcription reactions with diluted amounts of RNA polymerase (from 0.1 to 0.02 U of RNA polymerase per reaction mixture). Even at the lowest concentration of RNA polymerase, we could not detect any significant stimulation of transcription by TyrR and phenylalanine (data not shown). To test if histone-like DNA-binding protein HU (8) affects the transcription from the *mtr* or the *tyrP+3** promoter, we included purified HU protein in the transcription reaction mixtures. The results from these experiments are shown in Fig. 2. Addition of HU (400 nM) resulted in a significant reduction in transcription from both the *mtr* and *tyrP+3** promoters (Fig. 2A and B, lanes 2). This inhibition of transcription was relieved upon the addition of TyrR and phenylalanine (Fig. 2A and B, lanes 5). In contrast, in the absence of TyrR or phenylalanine, no significant stimulation of transcription was seen in either case (Fig. 2A and B, lanes 3 and 4). In the experiment with the *mtr* template, we also tested the effect of tryptophan on activation. As can be seen in Fig. 2A, lanes 8 and 9, in the presence of TyrR and tryptophan, but not in the presence of tryptophan alone, the transcription inhibition of the *mtr* promoter was partially lifted. This result is consistent with the previous finding that, in vivo, tryptophan can mediate transcription activation of the *mtr* promoter by the TyrR protein, although to a lesser degree than phenylalanine or tyrosine. However, in contrast to what we have observed in vivo, in the experiments involving both the *mtr* and the *tyrP+3** templates, no stimulation of transcription by tyrosine and TyrR was seen (results not shown).

We also tested the ability of the mutant TyrR protein, TyrR-RQ10, which is defective in activation in vivo (34), to activate transcription in vitro. In this experiment, we showed clearly that TyrR-RQ10 is completely inactive in stimulating transcription from both the *mtr* and *tyrP+3** promoters (Fig. 2A and B, lanes 7), indicating that an intact transcription activation domain is required for TyrR to counteract the HU-medi-

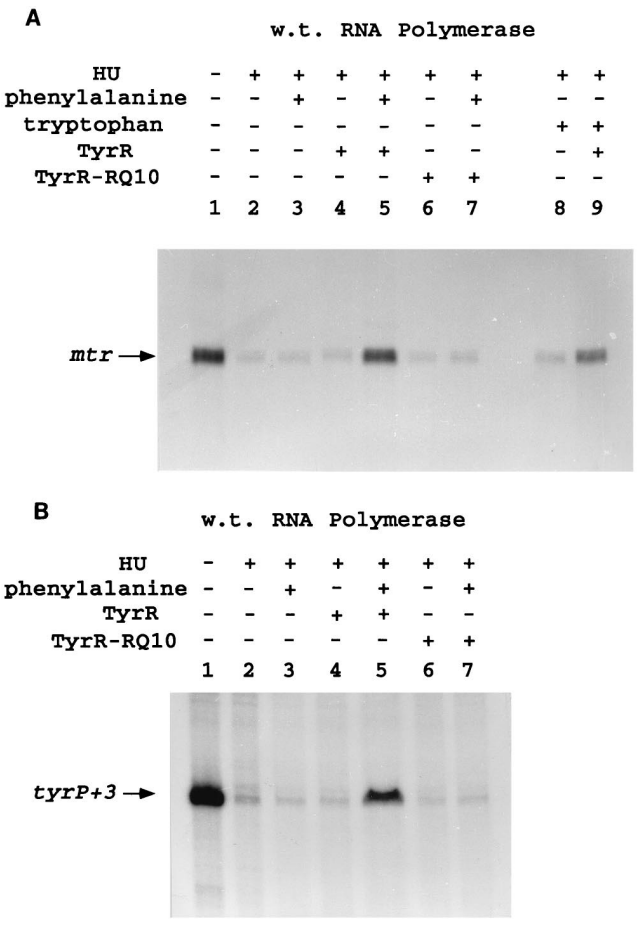

FIG. 2. (A) In vitro transcription from the *mtr* promoter in the presence of HU. Single-round in vitro transcription was performed as described in the legend to Fig. 1A. When added, the final concentrations of HU, TyrR-RQ10, and tryptophan were 400 nM, 200 nM, and 1 mM, respectively. w.t., wild type. (B) In vitro transcription from the *tyrP+3* promoter in the presence of HU. Single-round in vitro transcription was performed as described in the legend to Fig. 2A. A total of 0.015 pmol of DNA template (pDD3-tyrP+3*) was used for each transcription reaction mixture.

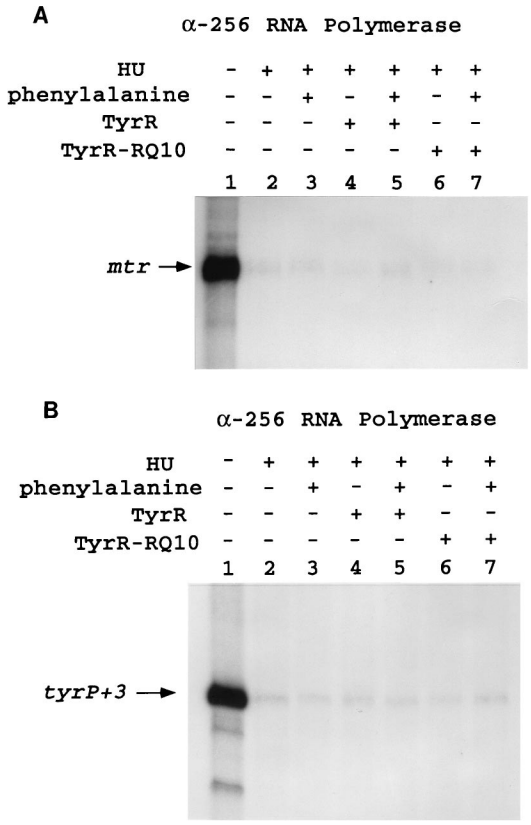

FIG. 3. (A) In vitro transcription from the *mtr* promoter by the mutant RNA polymerase (α-256). Single-round in vitro transcription was performed as described in the legend to Fig. 1A. A total of 0.5 pmol of mutant RNA polymerase (α-256) was used for each reaction mixture. HU was added to a final concentration of 400 nM. (B) In vitro transcription from the *tyrP+3* promoter by mutant RNA polymerase (α-256). Single-round in vitro transcription was performed as described in the legend to Fig. 3A. A total of 0.015 pmol of DNA template (pDD3-tyrP+3*) was used for each transcription reaction mixture.

ated transcription inhibition. To examine whether the C-terminal region of the RNA polymerase α subunit is required for alleviation of the HU-mediated transcriptional inhibition, we carried out in vitro transcription experiments with mutant RNA polymerase with a truncated subunit, α-256 (18). Two observations were made from these experiments. (i) HU inhibited transcription, from both the *mtr* promoter (Fig. 3A) and the *tyrP+3** promoter (Fig. 3B), with α-256 to the same extent as with the wild-type RNA polymerase. (ii) TyrR in combination with phenylalanine failed to relieve HU-mediated inhibition from both promoters when transcription was directed by α-256 (Fig. 3).

To determine the effect of HU on transcription of the *mtr* promoter on a linear DNA template, an in vitro transcription experiment was carried out with the 280-bp *Bam*HI fragment, purified from the plasmid pDD3-mtr. The addition of HU at a concentration of 400 nM did not significantly affect the transcription efficiency of the *mtr* promoter (results not shown). This indicates that the inhibitory effect of HU on *mtr* transcription is dependent on the superhelicity of the DNA template.

Effect of IHF on transcription from the *mtr* and *tyrP+3 promoters.** Next, we asked if host protein integration host factor (IHF), which is closely related to HU but binds to DNA in a sequence-specific manner, can inhibit transcription initiation from the *mtr* or the *tyrP+3** promoter. We showed that at a high concentration of IHF (400 nM), transcription initiation from both the *mtr* promoter and the *tyrP+3** promoter was greatly inhibited (Fig. 4A, lane 2, and B, lane 1). The levels of IHF-mediated transcriptional inhibition were also shown to be proportional to the levels of IHF present in the reaction mixtures, and below the concentration of 50 nM, no inhibition of transcription could be seen (data not shown). The inhibition of transcription by IHF was relieved by the presence of TyrR in combination with phenylalanine, but not by the presence of TyrR or phenylalanine alone nor by the presence of mutant TyrR protein TyrR-RQ10 with or without phenylalanine (data not shown). As occurred in the case of HU, the IHF-mediated transcription inhibition of both the *mtr* and *tyrP+3** promoters could not be lifted by the TyrR protein in the presence of tyrosine (data not shown).

Furthermore, in the transcription assay with the mutant RNA polymerase α-256, we showed that, regardless of the presence of phenylalanine, the TyrR protein was unable to stimulate transcription from the IHF-inhibited *mtr* or *tyrP+3** promoter (data not shown).

IHF does not protect the regulatory regions of *mtr* and *tyrP+3 from digestion by DNase I.** We scanned the *mtr* and *tyrP+3** promoter sequences with the MacTargsearch program

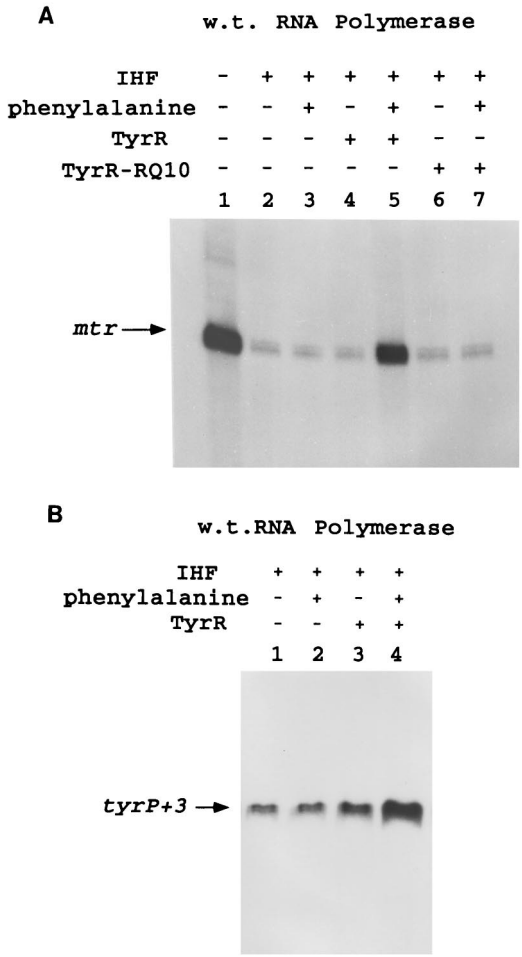

FIG. 4. (A) In vitro transcription from the *mtr* promoter in the presence of IHF. Single-round in vitro transcription was performed as described in the legend to Fig. 1A. IHF was added to a final concentration of 400 nM. w.t., wild type. (B) In vitro transcription from the *tyrP+3* promoter in the presence of IHF. Single-round in vitro transcription was performed as described in the legend to Fig. 1B. IHF was added to a final concentration of 400 nM.

(13) to see if there are any IHF binding sites. In each case, at least one weak but possible IHF binding site, which overlaps the strong TyrR box region, was found. To test if IHF can bind specifically to these putative target sites, we performed DNase I footprinting experiments. The *Bam*HI fragments which contain the *mtr* and *tyrP+3** promoter sequences were purified from plasmids pDD3-mtr and pDD3-tyrP+3* and were subjected to DNase I digestion in the presence of 40 or 400 nM IHF. In both cases, no footprint was seen, regardless of the concentration of IHF used (data not shown). This suggests that IHF does not bind specifically to the linear DNA sequences of the *mtr* and *tyrP+3** promoters.

In vivo role of HU in transcription from the *mtr* promoter. Experiments were carried out to see if the effects on transcription from the *mtr* promoter would corroborate those obtained in vitro and would support the notion that HU plays an important role in maintaining the correct conformation of the *mtr* promoter.

A strain completely lacking the DNA-bending protein HU can be constructed by transduction. The allele *hupA*::Cm^r is first introduced, followed by the allele *hupB*::Km^r (16). However, such *hupA hupB* double mutants have little viability and are cold sensitive.

Such *hupA hupB* derivatives of a set of *tyrR*$^+$ *trpR*$^+$, *tyrR366 trpR*$^+$, and *tyrR*$^+$ *trpR lac*Δ strains were constructed. These strains and their respective parents were then used as recipients in transformations with pMU3190, a low-copy Tp^r plasmid carrying a transcriptional fusion (35), so that β-galactosidase activity could be used as an index of *mtr* expression.

Transformants were selected on minimal medium supplemented with glucose, thiamine, histidine, proline, arginine, isoleucine, leucine, and valine; 25 μg of the chromogenic lactose analog X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) per ml; and 10 μg of trimethoprim per ml. Transformants were successfully isolated for the three parental strains and for the *hupA hupB tyrR*$^+$ *trpR*$^+$ and *hupA hupB tyrR366 trpR*$^+$ strains. The *hupA hupB* transformants grew very slowly on minimal medium and seemed to be more sensitive to trimethoprim than the parent strains. Transformants of the *hupA hupB tyrR*$^+$ *trpR* strain were successfully isolated by lowering the trimethoprim concentration to 1 μg/ml. The colonies were tiny, grew extremely slowly, and were an intense blue color.

Attempts were made to assay the β-galactosidase activity of the *hupA hupB* strains, but viability at the time of assay was only about 10%.

The *hup*$^+$ and *hupA hupB* derivatives of the three pairs of strains, carrying pMU3190, were streaked on a range of media containing X-Gal to obtain a relative measure of β-galactosidase activity. In each case, and on both minimal medium and Luria agar, the *hupA hupB* derivatives were a much more intense blue than their *hup*$^+$ parents. Since this result was apparent in the *trpR* background, the elevated expression of the *mtr-lac* fusion was not a consequence of loss of TrpR-mediated repression. It is also apparent that the normal level of IHF present in these *hup* mutants was unable to compensate for the loss of HU. Experiments in *himA himD* mutants also indicated that the absence of IHF had no significant effect on *mtr-lac* expression (results not shown), supporting the notion that IHF does not play a specific role in *mtr-lac* expression.

Discussion. Our results show that the transcription from both the *mtr* and the variant *tyrP* promoter *tyrP+3** can be influenced by the topological state of DNA, as has been shown in many other systems. Negative supercoiling strongly enhances transcription from the *mtr* and *tyrP+3** promoters. In vivo, bacterial DNA molecules (both chromosome and plasmid) are negatively supercoiled, and DNA supercoiling can stimulate transcription of some genes and inhibit transcription of others. Among the promoters whose expression can be stimulated by DNA negative supercoiling, the *E. coli lacP*^{s} and *tyrT* promoters have been studied extensively. Using a wide range of DNA templates containing the *lacP*^{s} promoter (relaxed and supercoiled), Borowiec and Gralla (3) have shown that within the physiological range, the rate of RNA polymerase-DNA open complex formation is proportional to the number of superhelical turns present in the plasmid templates. In the case of the *tyrT* promoter, Drew et al. (7) have demonstrated that negative supercoiling facilitates the unwinding of the DNA double helix preferentially around promoter regions.

With the inclusion of *E. coli* host factor HU or IHF, we are now able to demonstrate, in vitro, the magnitude of TyrR-mediated activation of both the *mtr* the *tyrP+3** promoters, which is comparable to that which we have seen in vivo. HU and IHF are small, basic, histone-like proteins which are abundant in the cell and which are involved in a number of cellular processes, such as site-specific recombination, replication, and transcription (8, 11, 17). These two proteins are structurally homologous and, in many cases, are functionally interchangeable (12, 17, 19). A common functional feature of HU and IHF is that they both bend DNA (15, 24, 28). In the case of the *mtr* and *tyrP+3** promoters, the HU- or IHF-mediated DNA bending could affect the promoter activities by introducing rate-limiting steps for transcription initiation. This idea is supported by our finding that $BaCl_2$, a chemical compound which is known to induce DNA bending (22), can also cause inhibition of transcription of both the *mtr* and *tyrP+3* promoters (results not shown). In vitro studies by Flashner and Gralla (9) have shown that $BaCl_2$ has the same inhibitory or stimulatory effects on the binding of a number of *E. coli* regulatory proteins to their DNA targets as HU. Furthermore, our observation that the $BaCl_2$-mediated inhibition of transcription can be alleviated by the presence of TyrR protein and phenylalanine (results not shown) implies that the mechanism by which the TyrR protein activates transcription of the *mtr* and *tyrP+3** promoters is to overcome the HU- or IHF-induced conformational change of DNA, which is undesirable for transcription initiation.

The in vivo results support the hypothesis that HU is involved in maintaining the correct tertiary structure of the *mtr* operator-promoter region, such that basal expression is reduced and strong activation by TyrR and tyrosine or phenylalanine is apparent.

Our in vitro transcription experiments involving the mutant TyrR protein TyrR-RQ10 or the mutant RNA polymerase α-256 show that both the N-terminal domain of TyrR and the C-terminal region of the α subunit of RNA polymerase are essential for activation. These results indicate that direct interactions between the N-terminal domain of TyrR and the C-terminal region of the α subunit of RNA polymerase are required for TyrR to counteract IHF- or HU-mediated transcription inhibition. Such interactions could function to recruit or stabilize RNA polymerase to the promoters and/or help to establish an adequate architecture at the promoter complexes for efficient transcription initiation.

The inability of IHF to protect the DNA fragments containing the *mtr* and *tyrP+3** regulatory regions from DNase I cleavage suggests that IHF does not bind specifically to these regions on linear templates. IHF is a sequence-specific DNA-binding protein which recognizes the core consensus sequence 5′-WATCAANNNNTTR-3′ (10), whereas HU binds to DNA in a sequence-nonspecific manner. However, IHF and HU share extensive sequence homology, especially in their DNA binding domains, and both proteins are believed to interact with DNA in the minor groove (32). In the case of lambda-

specific recombination, IHF (as an architectural element) is required, together with integrase and the attachment site, to form a highly ordered protein-DNA complex known as an intasome (27). Recently it has been reported by Segall et al. (27) that HU, as well as the eukaryotic DNA-binding (nonspecific) and -bending proteins HMG1 and HMG2, can replace IHF in the formation of stable intasomes. In addition, using a DNA template containing a mutant attachment site (*attL*QH′) in which the IHF binding site has been disrupted, Segall et al. have demonstrated that IHF, with integrase, is still able to form a stable intasome (27). It was thus suggested that IHF can bend DNA when it binds to DNA nonspecifically.

Using an in vitro transcription system, we have demonstrated both the phenylalanine- and the tryptophan-mediated activation of the *mtr* promoter and the phenylalanine-mediated activation of the *tyrP+3** promoter. However, despite various efforts, we were unable to show tyrosine-mediated activation of either the *mtr* or *tyrP+3** promoter. In vitro binding studies with purified TyrR protein by Davidson and coworkers (30) showed that the TyrR protein contains two aromatic amino acid binding sites, one ATP dependent and the other ATP independent. The ATP-dependent aromatic amino acid binding site, which has a higher affinity for tyrosine than for phenylalanine and tryptophan, is located in the central domain of TyrR and is responsible for tyrosine-mediated repression of the TyrR regulon genes, except *aroP* (21, 31). In contrast, the ATP-independent aromatic amino acid binding site has similar affinities for phenylalanine and tryptophan, but has no detectable affinity for tyrosine. The binding of the aromatic amino acid to this site has been proposed to be responsible for the phenylalanine- or tryptophan-mediated activation of the *mtr* promoter and the phenylalanine-mediated activation of the *tyrP* promoter (30). The failure to biochemically define a tyrosine binding site of TyrR which is accountable for tyrosine-mediated activation and our inability to demonstrate tyrosine-mediated activation in vitro raise the possibility that an additional cellular component(s) is involved in the tyrosine-mediated activation. Further genetic and biochemical studies are required to test this hypothesis.

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