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Role of integration host factor in the regulation of the *glnHp2* promoter of *Escherichia coli*

(transcription/σ^{54}/NR_I/nitrogen regulation)

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ABSTRACT **The *glnHPQ* operon of *Escherichia coli* encodes components of the high-affinity glutamine transport system. One of the two promoters of this operon, *glnHp2*, is responsible for expression of the operon under nitrogen-limiting conditions. The general nitrogen regulatory protein (NR_I) binds to two overlapping sites centered at −109 and −122 from the transcription start site and, when phosphorylated, activates transcription of *glnHp2* by catalyzing isomerization of the closed σ^{54}–RNA polymerase promoter complex to an open complex. The DNA-bending protein integration host factor (IHF) binds to a site immediately upstream of *glnHp2* and enhances the activation of open complex formation by NR_I phosphate. The NR_I-binding sites can be moved several hundred base pairs further upstream without altering the ability of NR_I phosphate to activate open complex formation. However, in this case, IHF diminishes open complex formation. We propose that the IHF-induced bend can facilitate or obstruct the interaction between NR_I phosphate and the closed complex depending on the relative positions of NR_I phosphate and σ^{54}–RNA polymerase on the DNA.**

Activation of transcription of nitrogen-regulated operons in *Escherichia coli* and other enteric bacteria requires RNA polymerase σ^{54} ($E\sigma^{54}$) and the *glnG* gene product NR_I in its phosphorylated active form (1, 2). In the well-studied *glnAp2* promoter, NR_I phosphate binds to upstream enhancer-like sequences and catalyzes the isomerization of a preexisting $E\sigma^{54}$–promoter closed complex to the open form (3, 4). Recent studies have provided evidence that bound NR_I phosphate makes contacts with the $E\sigma^{54}$–promoter closed complex through the formation of a DNA loop (5, 6).

Transcription of the *nif* operons of *Klebsiella pneumoniae* is also dependent on $E\sigma^{54}$ (7). The activator protein NIFA also binds to upstream sites (8, 9) and is functionally and structurally similar to NR_I (10, 11). In contrast to NR_I, NIFA has not been purified in active form (12, 13). Integration host factor (IHF) binds just upstream from the *nifH* and *nifU* promoters and stimulates NIFA-mediated activation (13–15). IHF is a sequence-specific DNA-bending protein, which is involved in gene expression and other processes in *E. coli* and some of its bacteriophages and plasmids (16).

The *glnHPQ* operon of *E. coli*, which encodes the components of the high-affinity glutamine transport system, is among the operons whose expression is induced under nitrogen-limiting conditions (17, 18). A promoter with homology to the σ^{54} promoters, *glnHp2*, has been identified (19). In this study, we present evidence for the existence of overlapping binding sites for NR_I upstream from the *glnHp2* promoter. We also found that IHF binds between the *glnHp2* promoter and the NR_I binding sites. This system allowed us to study the role of IHF in the activation of transcription by NR_I by using purified components.

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MATERIALS AND METHODS

Proteins, Primers, and Materials. Core RNA polymerase, σ^{54}, NR_I, and NR_{II} were purified as described (1, 20, 21). IHF was a gift from C. Robertson and H. Nash (National Institutes of Health). Primers FC5 (5′-CCACATCATCACACAATCG-3′), FC6 (5′-CAGACTTCATAGCATTTCC-3′), and FC7 (5′-GCATCTTCAGGGTATTGCC-3′) hybridizing at −217, +50, and −103 (5′ position), respectively, and primer FC1 (5′-GCGAGAGATATTCGTGG-3′), which hybridizes to T7 sequences close to the *Hin*dIII site of plasmid pTE103 (22), were synthesized at the Biopolymers Laboratory, Howard Hughes Medical Institute, Massachusetts Institute of Technology. The following materials were used: DNase I, *Mae* II, alkaline phosphatase, and bovine serum albumin, from Boehringer Mannheim; Klenow and other restriction endonucleases or DNA modifying enzymes, from New England Biolabs; radiolabels and Protosol, from DuPont/NEN; ultrapure solution ribonucleotides and Sephadex G-25, from Pharmacia LKB.

Construction of Plasmids. All transcription templates were derived from plasmid pTE103, which contains the multicloning site from pUC8 placed upstream from a bacteriophage T7 transcriptional terminator (22). Plasmid pFC50 was constructed by inserting the 540-base-pair (bp) *Eco*RV/*Sac* II fragment from pTN240 (18), into the *Sma* I site of pTE103. The sticky ends of this fragment and of those mentioned below were made blunt by using T4 DNA polymerase. The 540-bp fragment contains the *glnHp2* promoter with the upstream regulatory sequences and 66 nucleotides of the *glnH* coding region. Plasmid pFC54 was constructed by inserting the 180-bp *Mae* II/*Sac* II fragment from pTN240 (see position of the *Mae* II site in Fig. 4), which contains *glnHp2* and the IHF binding site but not the NR_I binding sites, into the *Hin*cII site of pTE103. Plasmid pFC55b was constructed by inserting the 360-bp *Eco*RV/*Mae* II fragment from pTN240, containing the NR_I binding sites of the *glnHp2* promoter region (see Fig. 4), into the *Sma* I site of pTE103. The orientation of the 360-bp fragment in pFC55b is opposite that in pFC50. Plasmid pFC57 was constructed by ligating the 180-bp *Bam*HI/*Hin*dIII fragment from pFC54 to plasmid pFC55b cut with *Bam*HI and *Hin*dIII. The NR_I binding sites in plasmid pFC57 are 273 bp further upstream than in pFC50. Plasmids were purified with a "mini" kit from Qiagen (Studio City, CA). The orientation of the fragments in the recombinant plasmids was confirmed by DNA sequencing with a Sequenase kit (United States Biochemical). Transcription at *glnHp2* on plasmids pFC50, pFC54, and pFC57 would generate transcripts of 418 nucleotides.

Abbreviations: IHF, integration host factor; DMS, dimethyl sulfate.

Transcription Assays. Transcriptions were as described (23). Plasmid DNA was purified by centrifugation in CsCl/EtdBr gradients and DNA concentrations were determined by absorbance at 260 nm. The concentration of DNA templates in all experiments was 5 nM. Core RNA polymerase, σ^{54}, NR_{II}, and IHF were present at 25, 100, 15, and 50 nM, respectively, or as indicated. NR_I was added at the indicated concentrations. The same radiolabeled nucleotide ([α-^{32}P]UTP; 3000 Ci/mmol; 10 mCi/ml; 1 Ci = 37 GBq) was used in all the experiments. Electrophoresis of RNA in urea/acrylamide gels was as described (1). After autoradiography, bands were cut out from the dried gels and placed in vials containing 5 ml of Scinti VerseTM I (Fisher Scientific) and 0.3 ml of Protosol. Vials were incubated overnight at 37°C and assayed in a 1211 Rackbeta liquid scintillation counter (LKB).

Footprinting. DNA footprinting experiments were carried out on supercoiled DNA as described (24). Plasmid pFC50 was incubated with the proteins under the same conditions used for transcription. After 20 min of incubation at 37°C, the DNA was treated with DNase I, dimethyl sulfate (DMS), or potassium permanganate as described (refs. 24 and 25; S. Sasse-Dwight and J. D. Gralla, personal communication). Samples treated with DNase I or potassium permanganate were extracted with phenol/chloroform. DMS-treated DNA was cleaved with 1 M piperidine. All samples were then passed through Sephadex G-25 spin columns equilibrated in water; 5×10^5 cpm of ^{32}P-end-labeled FC5, FC6, or FC7 primer was added per reaction mixture. Each sample was analyzed by primer-extension analysis using alkaline denaturation as described (24, 26). Hybridizations to the FC5, FC6, and FC7 primers were carried out at 54°C, 52°C, and 55°C, respectively. Reaction products recovered by ethanol precipitation were analyzed together with dideoxynucleotide sequencing reaction products on 7% polyacrylamide gels containing 7 M urea.

RESULTS

Binding Sites for IHF and NR_I. Examination of the regulatory region of the *glnHPQ* operon (ref. 18; see Fig. 4) shows the presence of two putative IHF binding sites, one immediately upstream from the *glnHp2* promoter (−46 to −34; see Fig. 4) identical to the IHF DNA binding consensus (5′-A/TATCAAN$_4$TTA/G-3′) (16, 27) and another one further upstream (−158 to −146) with one mismatch. DNase I protection patterns of IHF on both strands of the *glnHp2* promoter region were obtained (Fig. 1*A*, top strand; Fig. 1*B*, bottom strand). IHF protected the site close to *glnHp2* but not the other site. The protected region is extensive, approximately from −60 to −24 on both strands (Fig. 1 *A* and *B*), considering the small size of the IHF dimer (M_r 20,000) (16). We also probed specific interactions between IHF and *glnHp2* with DMS, a reagent that does not cleave the DNA backbone. Inhibition or enhancement of methylation can then be detected after chemical cleavage of the modified bases. IHF-dependent protection of the G residues at positions −50, −40, and −34 was observed (Fig. 2*B*, lanes 4 and 5). Furthermore, in the presence of IHF the reactivity of G residues at positions −57 and −39 and of A residues at −47 and −48 was enhanced.

It has been shown that sequences upstream from *glnHp2* play a role in the inducible expression of the *glnHPQ* operon under nitrogen-limiting conditions (18). By inspection of this region, we found four sequences that fit the consensus NR_I binding site 5′-TGCACCAN$_3$TGGTGCA-3′ (see Fig. 4). Sites 3 and 2 overlap and have only one and two mismatches, respectively. Sites 1 and 4 deviate from consensus by 3 bp, and the spacing in site 4 is four instead of three. Sites 1 and 2 also overlap. We carried out DNase I footprinting experiments to determine which sites are occupied by NR_I. In all the experiments in which NR_I was present, the kinase NR_{II} and ATP were added to allow phosphorylation of NR_I. As expected, sites 2 and 3 were protected at the low NR_I concentrations (25–50 nM), while sites 1 and 4 became occupied only at 200 nM NR_I (Fig. 1*C*). The interaction of NR_I with its binding sites was also studied on intact plasmid DNA by DMS footprinting. The pattern of methylation at both strands is shown in Fig. 2 *A* and *B*. In the presence of 25–50 nM NR_I, protection of G residues in sites 2 and 3 only was observed; −102, −112 to −115, −125, and −126 in the bottom strand (Fig. 2*A*, lanes 4 and 5), and −105, −106, −118, and −119 in the top strand (Fig. 2*B*, lane 2). G residues at −128 in the bottom strand (Fig. 2*A*, lane 5) and at −103 in the top strand (Fig. 2*B*, lane 2) were hypermethylated. At a high concentration of NR_I (200 nM), sites 1 and 4 also became occupied; positions −72, −76, and −139 on the bottom strand (Fig. 2*A*, lane 6) and −87, −131, and −142 on the top strand (Fig. 2*B*, lane 3) were protected from DMS attack while positions −84, −86, −141, and especially −130 on the bottom strand, and −73, −75, and −144 on the top strand were hyperreactive. The hyperreactive bands may reflect both a bending of the DNA induced by the bound NR_I and a distortion of the DNA between two occupied half sites (28).

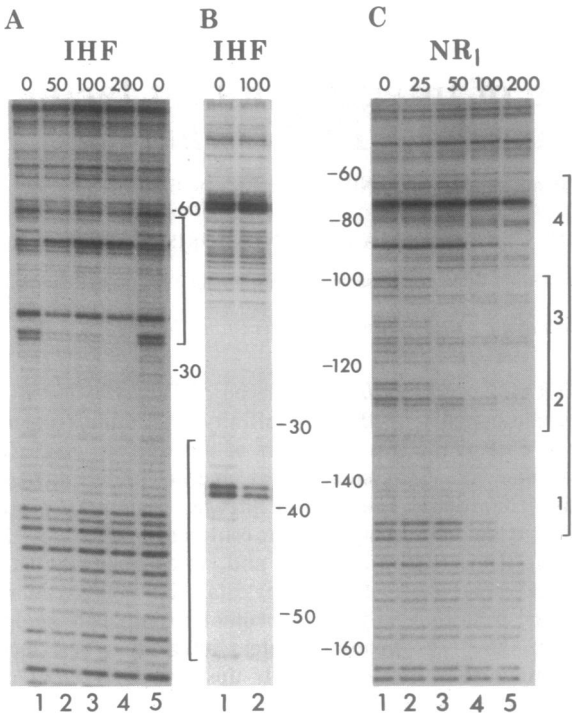

FIG. 1. DNase I footprinting of IHF and NR_I-binding sites in the *glnHp2* promoter regulatory region. Plasmid DNA was incubated with increasing concentrations of IHF (*A* and *B*) or NR_I (*C*), shown above each lane (nM). Primer extensions were carried out with primer FC6 (*A*) for the top strand and primer FC7 (*B*) or FC5 (*C*) for the bottom strand. The extent of protection by IHF or NR_I is indicated by brackets; in the case of NR_I, the small and the large brackets indicate protection at 50 nM and 200 nM, respectively. The sequence and the NR_I-binding sites are numbered as shown in Fig. 4.

Closed and Open Complexes at the *glnHp2* Promoter. The *glnHp2* promoter has a T residue at position −14 instead of the G in the GC doublet found in all previously studied σ^{54}-dependent promoters (7, 29). We used DMS footprinting to determine whether or not closed complexes are formed at this unusual promoter. Fig. 2*C* shows the analysis of the bottom strand of *glnHp2*. The interaction of Eσ^{54} with the promoter was observed in the absence of NR_I phosphate (compare lanes 1 and 2); G residues at −28, −24, −22, and −13 were protected from methylation, indicating that Eσ^{54}

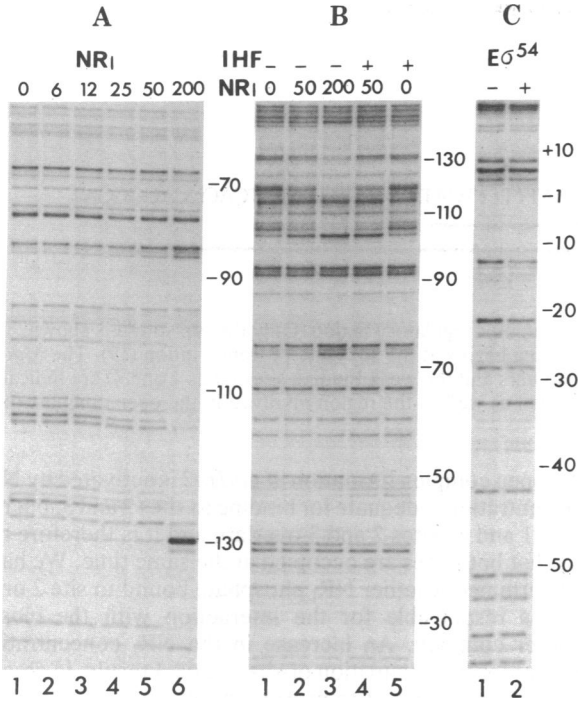

FIG. 2. DMS footprints of NR_I, IHF, and $E\sigma^{54}$ interactions with the *glnHp2* promoter region. (*A*) Methylation pattern at NR_I-binding sites on the bottom strand. (*B*) Methylation pattern at NR_I (lanes 1–4) and IHF (lanes 4 and 5) binding sites on the top strand. Numbers above each lane indicate concentration of NR_I (nM). (*C*) Methylation pattern of the closed complex on the bottom strand. The presence (+) or absence (−) of IHF or $E\sigma^{54}$ in each incubation mixture is indicated. Concentrations were as follows: E, 25 nM; σ^{54}, 100 nM; IHF, 50 nM; NR_I, as indicated. The sequence is numbered as shown in Fig. 4. Extensions were carried out with primers FC5 (*A*), FC6 (*B*), and FC7 (*C*).

forms a closed promoter complex with *glnHp2*. Protection of these residues was unchanged when NR_I and NR_I plus IHF were also present (data not shown). A number of residues in the region between −6 and +3 became reactive to DMS in the presence of $E\sigma^{54}$ and NR_I, and they became even more reactive when IHF was present (data not shown). The reactivity of residues flanking the transcription start site in the presence of NR_I phosphate may reflect formation of the open promoter complex.

Open complexes between $E\sigma^{54}$ and the *glnAp2* promoter have been visualized with potassium permanganate (28), which oxidizes preferentially T and C residues in single-stranded DNA. We therefore used this reagent to probe *glnHp2* (Fig. 3*A*, bottom strand; Fig. 3*B*, top strand). In the presence of $E\sigma^{54}$ and NR_I phosphate, T residues at −12, −10, −8, −3, −2, and +8 and a C residue at −4 on the bottom strand (Fig. 3*A*, lanes 4–6), and T residues at −9, −7, −5, −1, and +2 on the top strand (Fig. 3*B*, lanes 4–7) became hyperreactive. These residues were not reactive when NR_I was absent (Fig. 3, lanes 2). This region therefore represents the DNA melted in an open complex (Fig. 4). When NR_I was present at a high concentration (200 nM), there was less open complex formation (compare lanes 4 and 6 in Fig. 3 *A* and *B*). On the other hand, the same residues became even more reactive when IHF was present (compare lanes 4 and 5 in Fig. 3*A* and lanes 4 and 5 and lanes 6 and 7 in Fig. 3*B*) but did not react when only IHF or IHF and $E\sigma^{54}$ were present, indicating that IHF stimulates the NR_I-dependent open complex formation. The results of the footprinting experiments are summarized in Fig. 4.

IHF Affects Activation of *glnHp2* Transcription by NR_I. All the DNA templates used in these experiments carried the

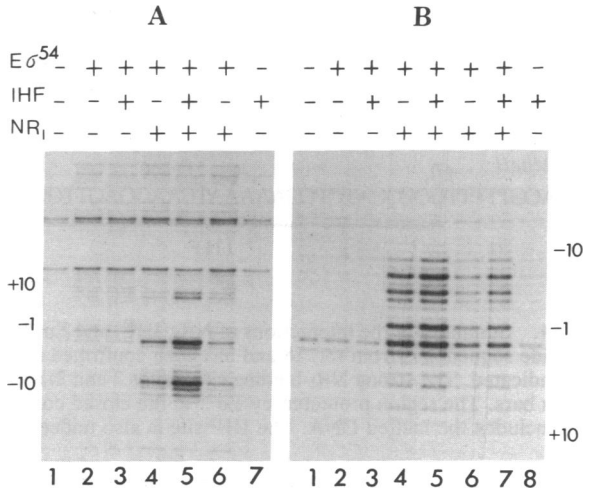

FIG. 3. Detection of the open complex at *glnHp2* with potassium permanganate. Supercoiled DNA was treated with potassium permanganate after incubation in the presence (+) or absence (−) of proteins as indicated above each lane. Concentrations of proteins when present were as follows: E, 25 nM; σ^{54}, 100 nM; IHF, 50 nM; NR_I, 50 nM (lanes 4 and 5) and 200 nM (lanes 6 and 7*B*). The sequence is numbered as shown in Fig. 4. Primer extensions were carried out with primer FC7 for the bottom strand (*A*) and primer FC6 for the top strand (*B*).

glnHp2 promoter and the IHF-binding site and included the following: pFC50, which contains the NR_I-binding sites in the wild-type position—that is, the center of sites 2 and 3 are at −122 and −109, respectively; pFC54, which has NR_I-binding sites removed; and pFC57, which contains the NR_I-binding sites further upstream in inverted orientation—that is, the center of sites 2 and 3 are at positions −382 and −395, respectively.

Using the wild-type promoter, we first showed that NR_I phosphate was able to activate initiation of transcription at *glnHp2* (Fig. 5, pFC50, lanes 1–6). The concentration of NR_I needed for activation was ≈5 times higher than that needed for activation of *glnAp2* (23). When the NR_I concentration was higher than 50 nM, the activation was decreased (Fig. 5, pFC50, lanes 5 and 6). In the presence of IFH, the activation by NR_I, at concentrations of 10–200 nM, was stimulated (Fig. 5, pFC50, lanes 7–12). IHF by itself did not activate transcription (Fig. 5, lane 7). The stimulation by IHF was greater at lower concentrations of NR_I (≈5-fold at 10 nM NR_I) than at higher concentrations (2-fold at 50 nM NR_I) (Fig. 5, pFC50). On the other hand, the stimulation by IHF was the same over a wide range of $E\sigma^{54}$ concentrations (data not shown). The stimulatory effect could be observed at an IHF concentration of 15 nM but was maximal at 50 nM (data not shown). IHF stimulation was not observed in the NR_I-mediated activation of *glnAp2* (data not shown), a promoter that lacks an IHF-binding site.

When the NR_I-binding sites were removed, NR_I was able to activate transcription but only at the higher concentration (Fig. 5, pFC54). In this case, IHF inhibited the activation of *glnHp2* (Fig. 5, pFC54, lanes 7–12). When the NR_I-binding sites were moved further upstream, NR_I was still able to activate transcription at a low concentration (Fig. 5, pFC57, lanes 1–6) as has been shown for *glnAp2* (3, 30); however, in this case too, IHF inhibited the activation (Fig. 5, pFC57, lanes 7–12).

DISCUSSION

The *glnHp2* promoter differs from all previously identified σ^{54}-dependent promoters by having the dinucleotide TC rather

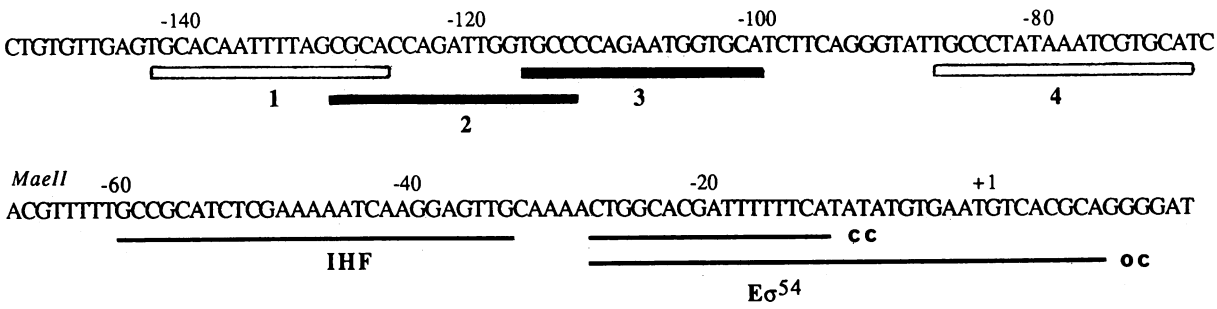

FIG. 4. Summary of the interactions of NR_I, IHF, and $E\sigma^{54}$ with the *glnHp2* promoter regulatory region. Only the top strand is shown. The nucleotide sequence is from ref. 18 and has been confirmed in our study. Numbering begins at the start site of transcription (19). The *Mae* II site is indicated. The strong NR_I-binding sites (sites 2 and 3) are indicated by solid bars and the weak binding sites (sites 1 and 4) are indicated by open bars. The region protected by $E\sigma^{54}$ in the closed complex (cc) is underlined as well as the region involved in the open complex (oc), which includes the melted DNA. The IHF site is also underlined.

than GC at position −13 (Fig. 4). We have now shown that this promoter, like *glnAp2* (28, 30) but unlike *nifHp* (11), can interact with $E\sigma^{54}$ to form a closed complex (Fig. 2*C*). The −15 to −19 region of the *glnHp2* promoter, like that of *glnAp2*, is rich in T residues while that of *nifHp* is rich in C residues. The idea that this feature of the promoter may increase its affinity for $E\sigma^{54}$ is supported by the observation that a mutant *nifH* promoter with three of the C residues converted to T residues is capable of forming a closed complex (11).

The binding of IHF to its site located just upstream of *glnHp2* does not affect the formation of the closed complex (data not shown). Neither does the binding of IHF affect the ability of NR_I or NR_I phosphate to bind to its sites (Fig. 2*B*; data not shown) located further upstream (Fig. 4). IHF by itself is not able to stimulate the isomerization of the closed complex to the open form (Fig. 5). This transition requires NR_I phosphate, but it can be enhanced or diminished by IHF, depending on the position of the NR_I-binding sites relative to the promoter.

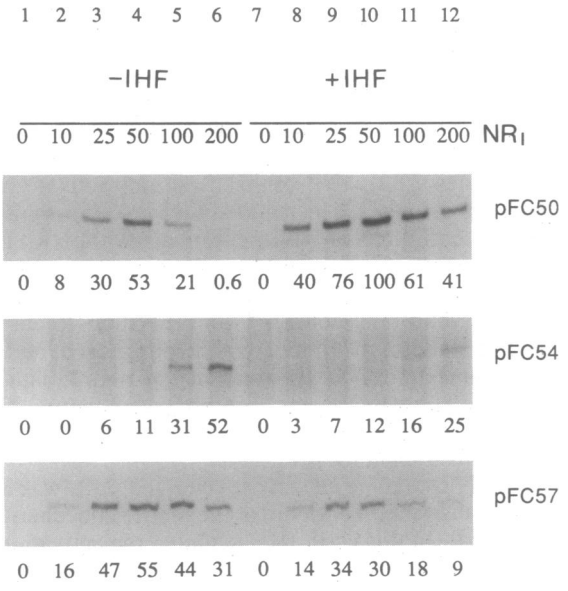

FIG. 5. Activation of transcription from *glnHp2* by NR_I phosphate in the absence or presence of IHF. Template pFC50 contains the wild-type *glnHp2* promoter region, pFC54 is like pFC50 but without the NR_I-binding sites, and pFC57 contains the NR_I-binding sites placed 273 bp further upstream. All reaction mixtures contained 25 nM E, 100 nM σ^{54}, 15 nM NR_{II}, and 4 mM ATP. NR_I was added as indicated. When present, IHF was at a concentration of 50 nM. The numbers below each lane represent the percentage of transcription activation relative to the amount of transcription obtained with pFC50 in the presence of 50 nM NR_I and IHF.

The open complex formation at *glnHp2* is activated by NR_I at concentrations adequate for binding to sites 2 and 3, but not to sites 1 and 4. Sites 2 and 3 overlap, and it is therefore not likely that both sites are occupied at the same time. We have not determined whether NR_I phosphate bound to site 2 or to site 3 is responsible for the interaction with the closed promoter complex. An increase in the NR_I concentration sufficient for the occupation of sites 1 and 4 results in greatly decreased activation of transcription. However, NR_I in this high concentration is effective in the activation of transcription on a template whose NR_I-binding sites have been removed (Fig. 5). We may therefore conclude that the ability of NR_I phosphate to bring about the isomerization of the closed complex to the open form is increased by its binding to site 2 or 3 and is decreased by its binding to site 1 or 4.

IHF, at a concentration adequate for maximal occupation of its binding site, increases the ability of NR_I phosphate bound to site 2 or 3 to activate the formation of the open complex on the template where these sites are located at their normal distance from the promoter (Fig. 5). This stimulation by IHF is most apparent at a NR_I concentration insufficient for full occupation of the binding sites. Moving the NR_I-binding sites several hundred base pairs further upstream does not diminish the ability of NR_I phosphate bound to site 2 or 3 to activate transcription, but in this case IHF not only fails to increase the activation of transcription but actually decreases it significantly. Similar results were obtained when a template lacking NR_I-binding sites was used. In this case, a high concentration of NR_I was required for activation, and IHF inhibited the activation (Fig. 5).

We interpret these results according to the previously established ability of IHF to bend DNA by more than 140° (31, 32). The ability of NR_I phosphate bound to sites far from the promoter to activate the open complex formation depends on the flexibility of the DNA, which allows the NR_I phosphate to contact the closed promoter complex. The bend in the DNA induced by binding of IHF to its site close to the promoter would increase the probability of a fruitful contact between NR_I phosphate and the closed complex when NR_I phosphate is properly spaced with regard to the promoter. The bend in the DNA induced by the binding of IHF would, however, diminish the flexibility of the DNA and therefore interfere with the ability of NR_I phosphate to activate open complex formation from binding sites whose position has been altered. The fact that IHF interferes with the activation of transcription by NR_I phosphate on a template lacking NR_I-binding sites could be explained by the assumption that in this case NR_I phosphate exerts its effect by binding to nonspecific sites on the DNA (5, 33, 34).

The results of our investigation using purified components support and extend the conclusions reached by Hoover *et al.* (15) on the role of IHF in the activation of transcription of the

nifH promoter by NIFA. These results may also explain the observation that in intact cells the NR_I binding sites of the IHF-independent *glnAp2* promoter can be moved more than 1 kilobase in either direction and remain functional, while in the case of *nifH*, where activation is stimulated by IHF, a corresponding change reduces transcription 10-fold (8). Our results suggest that a DNA-bending protein could stimulate or diminish activation of gene expression without interacting directly with the activator or with the RNA polymerase. This mechanism may play a role in the regulation of gene expression in eukaryotic cells where some transcription factors have been shown to bend DNA (35–37).

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