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Activation of *glnA* Transcription by Nitrogen Regulator I (NR_I)-Phosphate in *Escherichia coli*: Evidence for a Long-Range Physical Interaction between NR_I-Phosphate and RNA Polymerase

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Growth of cells of *Escherichia coli* in nitrogen-limited medium induces the formation of glutamine synthetase, product of the *glnA* gene, and of other proteins that facilitate the assimilation of nitrogen-containing compounds. Transcription from the *glnAp2* promoter of the *glnALG* operon requires the phosphorylation of nitrogen regulator I (NR_I) and, for optimal transcription, the binding of NR_I-phosphate to two sites that can be over 1,000 base pairs from the binding site for RNA polymerase. In other procaryotic genes, placement of an activator-binding site further upstream from the start site of transcription diminishes expression. To determine how NR_I-phosphate activates transcription and why NR_I-dependent transcription differs from activation in other systems, we constructed recombinant plasmids with small alterations between the binding sites for NR_I-phosphate and RNA polymerase and between the two high-affinity NR_I-binding sites. We demonstrate that tightly bound NR_I-phosphate activated transcription from either side of the DNA helix when at least 30 base pairs separated NR_I-phosphate from RNA polymerase. In contrast, activation from a partial NR_I-binding site was effective only from one side of the DNA. We also observed that *glnA* expression was optimal when the two high-affinity NR_I-binding sites were on the same side of the DNA helix. We explain these results on the basis of a hypothesis that a contact between RNA polymerase and NR_I-phosphate bound to an upstream site determines the rate of *glnA* transcription.

Among nitrogen sources, ammonia supports the fastest growth of cells of *Escherichia coli* and *Salmonella typhimurium*. Growth on other sources of metabolizable nitrogen, such as histidine or glutamine, is nitrogen limited and induces the synthesis of glutamine synthetase, the product of the *glnA* gene, and of other proteins that accelerate transport or degradation of nitrogen-containing compounds (reviewed in reference 28). The first operon activated during the transition to nitrogen-limited growth is the *glnALG* operon. Transcription from *glnAp2*, the major promoter of the *glnALG* operon, requires the phosphorylated form of NR_I (nitrogen regulator I), product of the *glnG* (or *ntrC*) gene, and σ^{54}, product of the *rpoN* (or *ntrA*) gene (9, 10, 14, 19, 26, 28). Nitrogen regulator II, the product of the *glnL* (or *ntrB*) gene, phosphorylates NR_I during nitrogen-limited growth and dephosphorylates NR_I-phosphate when ammonia is in the growth medium (12, 19).

Core RNA polymerase complexed with σ^{54} transcribes many nitrogen-regulated genes and recognizes promoters with a consensus sequence CTGGYAYR-N_4-TTGCA instead of promoters with the sequence of the canonical promoter of enteric bacteria, TTGACA-N_{17}-TATAAT (1, 9, 10). Core RNA polymerase complexed to σ^{54} protects DNA in the vicinity of *glnAp2* against DNaseI digestion from 45 bases upstream to 10 bases downstream of the start of transcription (9, 20, 31). The binding of σ^{54}-RNA polymerase to *glnAp2* DNA does not require and is not facilitated by NR_I or NR_I-phosphate (9, 20, 31). Variation of the intracellular concentration of σ^{54} does not regulate the induction of nitrogen-regulated proteins because the availability of nitrogen in the medium does not regulate the transcription of *rpoN* (6, 7).

Optimal transcription from *glnAp2* requires the binding of NR_I to specific sites upstream of the start site of transcription (Fig. 1; 13, 20, 27). The contacts made by NR_I in vivo and in vitro indicate that NR_I binds with high affinity to promoter-distal sites 1 and 2 and with moderate affinity to site 3. NR_I also binds weakly to sites 4 and 5 in vitro (9, 20) but not in vivo (31). Without the promoter-distal site 1, full stimulation of transcription from *glnAp2* requires a higher concentration of NR_I. NR_I can stimulate transcription from *glnAp2* even without both high-affinity sites 1 and 2, but expression is only 15% of optimal and requires a high concentration of NR_I. The absence of sites 3, 4, and 5 does not affect this residual NR_I-dependent activation (27).

Small increases in the distance between the binding sites for an activator and RNA polymerase diminish transcription of other positively regulated procaryotic genes (4, 15, 16). For these and other positively controlled procaryotic genes, the activator has been postulated to stimulate transcription by contacting RNA polymerase (4, 11, 23, 29). Unlike these other systems, the two high-affinity NR_I-binding sites can be 1,400 bases further upstream or downstream and still activate *glnAp2* optimally (27). The difference between activation by NR_I and by other procaryotic activators might be that NR_I activates the transcription of *glnAp2* by a different mechanism. To further understand how NR_I-phosphate stimulates transcription from *glnAp2* and to possibly determine the features that distinguish control of *glnA* expression from expression of other genes, we describe the effect of small positional alterations between the binding sites for RNA polymerase and NR_I and between the two high-affinity NR_I-binding sites.

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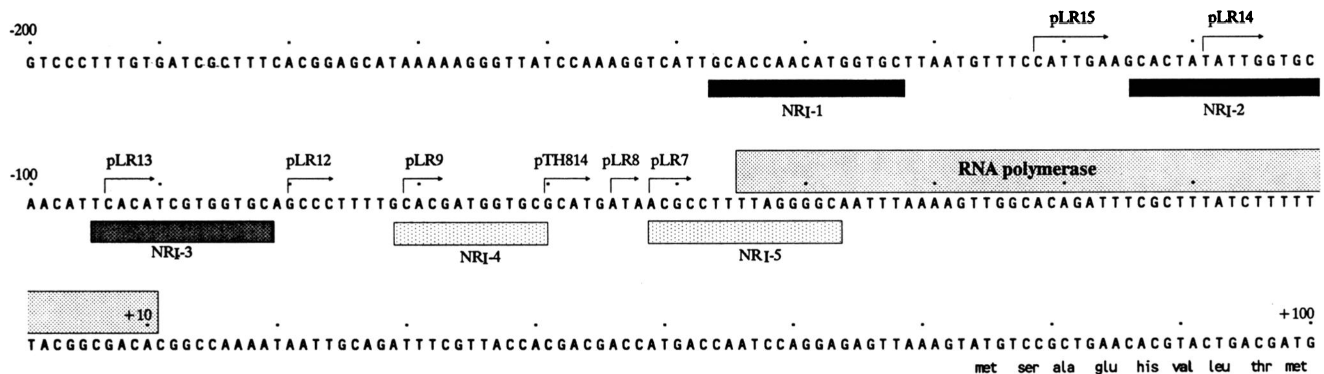

FIG. 1. Binding sites for NR$_I$ and RNA polymerase at the *glnAp2* promoter in *E. coli*. The sequence is from reference 26. The start site for transcription from *glnAp2* is designated +1. The high-affinity NR$_I$-binding sites 1 and 2, the lower-affinity site 3, and the very-low-affinity sites 4 and 5 are indicated. RNA polymerase protects about 55 bp of DNA from DNaseI digestion (20, 31), but the sequence similar to that from other nitrogen-regulated promoters (1) is from −27 to −11. The deletion endpoints used in construction of the recombinant plasmids discussed in this paper are indicated by arrows. Construction of the original deletion plasmids has been described previously (27).

MATERIALS AND METHODS

Strains, cell growth, and enzyme assays. Growth and harvesting of cells and all standard techniques involving nucleic acids have been described elsewhere (17, 30). The strains were derivatives of the *E. coli* K-12 strain YMC10 (*thi-1 endA1 hsdR17 lacU169 hutC$_k$*) (2), which is considered wild type. Strain YMC22 is also *rpoN208*::Tn*10*, and strain TH16 is also *glnA21*::Tn*5*.

The assay for glutamine synthetase activity has been described previously (30). The values for glutamine synthetase activity are the average of two to six determinations, each determination using an independent transformant. All activities reported here were normalized to the glutamine synthetase activity measured from cells of strain YMC10 with plasmid pLR146 after the corrections described below were subtracted. In cells with plasmids containing the *glnA* gene, transcription of *glnA* during nitrogen-limited growth is from the plasmid *glnAp2* promoter and from σ^{54}-independent promoters of the plasmid. The σ^{54}-independent expression of plasmid-borne *glnA* was determined by measuring glutamine synthetase activity from cells of strain YMC22 (σ^{54} deficient) and was subtracted from the value obtained from cells of strains YMC10 and TH16, all bearing the plasmid of interest. This correction is about 5% of the activity of glutamine synthetase measured from cells of strain YMC10(pLR146) (see Table 1 of reference 27). When the recombinant plasmids were carried in a wild-type strain, YMC10, glutamine synthetase activity was the result of transcription of *glnA* from the *glnAp2* promoter of the plasmid, σ^{54}-independent promoters of the plasmid, and the chromosomal *glnAp2* promoter. To correct for the chromosomal expression of *glnA*, the activity of glutamine synthetase from cells of the wild-type strain, YMC10, without a plasmid was subtracted from the total glutamine synthetase activity. This correction is valid because in cells of a wild-type strain that is lysogenic for a lambda bacteriophage, λ*glnl01*, which has a fusion of the *glnA* promoter region to *lacZ* (2), the presence of a high-copy plasmid bearing *glnA* does not affect the level of β-galactosidase (L. Reitzer, unpublished observation). This result means that the presence of multiple copies of a transcriptionally active *glnAp2* promoter does not affect expression from the chromosomal *glnAp2* promoter. This correction is about 5% of the activity measured from cells of strain YMC10(pLR146).

Plasmid constructions. The plasmids described in this work were constructed from plasmids with deletions into the *glnA* promoter region (27). The deletion endpoints (Fig. 1) are all upstream of the contacts made by RNA polymerase bound to DNA near the *glnAp2* promoter in *E. coli* (20, 31). The basic structures of the plasmids used for the constructions are shown in Fig. 2; construction of the plasmids is described in further detail in Tables 1 to 5 and in appropriate sections of Results. All but four of the plasmids contain the *Cla*I-*Pvu*II segment of pBR322 that has the *bla* gene. Four plasmids, pTH814, pLR121, pLR150, and pLR151 (Table 2 and Fig. 2B), have all of pBR322. Exclusion of the data obtained from cells with these four plasmids would not alter the conclusions of this paper.

The constructions generally resulted in the creation of a unique restriction site between two regions of interest. Synthetic oligonucleotides were inserted into these unique restriction sites to further alter the distance between the two regions of interest. For the purpose of these constructions, the use of *Kpn*I linkers was desirable. Cleavage of this site with the *Kpn*I and *Asp*718 restriction endonucleases leaves 4-base-pair (bp) 3′ and 5′ single-stranded overhangs, respectively. These staggered ends make the removal or addition of 4 bp straightforward. The destruction or recreation of restriction sites was verified. To check for small deletions or insertions, the recombinant plasmids were digested with *Hae*III, which cuts the DNA at over 20 locations. Plasmids were checked for the insertion of only a single oligonucleotide linker by the absence of an inappropriate restriction site. For example, tandem *Kpn*I linkers would create a *Sma*I site.

RESULTS

Expression of *glnA* from high-copy plasmids. Measurement of glutamine synthetase activity from cells with recombinant *glnA* plasmids was used to assess transcription initiated from *glnAp2* during nitrogen-limited growth. Glutamine synthetase activity has been shown to accurately reflect the expression of *glnA* (26). The recombinant *glnA* plasmids were placed into cells of strains YMC10 and TH16 to study the effect of two concentrations of NR$_I$ on transcription of *glnA* from the plasmid. Transcription of the *glnG* gene, which encodes NR$_I$, in cells of strain YMC10 initiates from the chromosomal *glnAp2* promoter and results in about 70 dimers of NR$_I$ per cell (21, 25). NR$_I$ at this level, which is

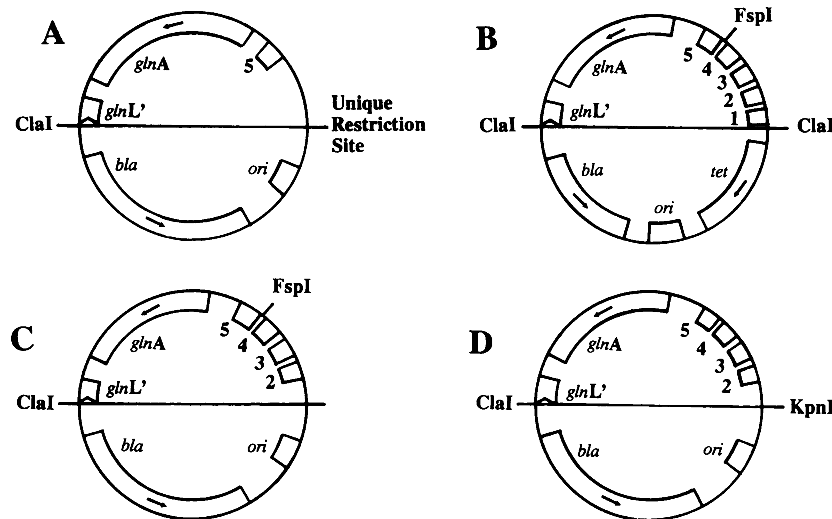

FIG. 2. Plasmid constructions. All plasmids constructed for this work contain a defined portion of pBR322, which is shown in the bottom half of the diagrams. For the plasmids in panels A, C, and D, the pBR322 portion contains 2,320 bases between the *Cla*I and *Pvu*II restriction endonuclease sites; in panel B, the plasmids represented contain all of pBR322. All plasmids also contain the region of the *glnALG* operon from 1,845 bases downstream to at least 52 bases upstream of the start site of transcription from the *glnAp2* promoter, which includes the entire *glnA* structural gene, 66 bases of the *glnL* structural gene, and the entire RNA polymerase-binding site for the *glnAp2* promoter. These figures are not drawn to scale. (A) All plasmids described in Table 2 (but for pTH814, pLR121, pLR150, and pLR151) and all plasmids described in Table 3 were constructed from a plasmid with the indicated structure. To construct plasmids in which the distance between the RNA polymerase-binding site and one or two high-affinity NR_I-binding site or sites was varied DNA containing a high-affinity NR_I-binding site or sites was inserted into the unique restriction endonuclease site. These constructions generally resulted in plasmids with a unique restriction site, which allowed further alteration of the distance between two sites of interest. (B) Plasmids pTH814, pLR121, pLR150, and pLR151, whose construction is described in Table 2, were derived from a plasmid with the indicated structure. Synthetic oligonucleotides were inserted into the *Fsp*I site to vary the distance between the RNA polymerase-binding site and the two high-affinity NR_I-binding sites. (C) To vary the distance between the RNA polymerase-binding site and a partial NR_I-binding site 2, synthetic oligonucleotides were inserted into the *Fsp*I site of plasmid pLR169, whose structure is shown. Construction of these plasmids is discussed in more detail in Table 4. (D) To vary the distance between two high-affinity NR_I-binding sites, DNA containing the high-affinity NR_I-binding site from the *glnL* promoter region was inserted into the unique *Kpn*I site of plasmid pBM8, whose structure is shown. More details of the constructions are given in Table 5.

considered a high concentration, almost completely occupies the high-affinity NR_I-binding sites 1 and 2 and partially occupies the low-affinity site 3 on recombinant plasmids (31). In cells of strain TH16, NR_I is considered to be at a low level

TABLE 1. Parental plasmids used to construct plasmids described in this work

Parental plasmid[a]	Derived from[b]:	Upstream boundary[c]	NR_I site(s) remaining
pBM1	pTH814	−60, *Xho*I	5
pBM8	pLR7	−52, *Kpn*I	5
pBM12	pLR15	−122, *Kpn*I	2–5
pLR9		−71, *Kpn*I	4, 5
pLR88	pLR8	−55, *Kpn*I	5
pLR145	pLR12	−80, *Kpn*I	4, 5
pLR146	pLR1	−540, *Kpn*I	1–5
pLR157	pLR13	−94, *Kpn*I	3–5
pLR168	pLR14	−109, *Kpn*I	3–5 and half of 2
pLR169	pLR14	−109, none	3–5 and half of 2

[a] All but pLR9 contain the portion of pBR322 from the *Cla*I to the *Pvu*II site that contains the *bla* gene. They were derived from plasmids indicated in the second column by digestion with restriction enzymes *Pvu*II and *Asp*718 (*Pvu*II and *Xho*I for pBM1), filling in with the Klenow fragment of DNA polymerase I of *E. coli*, isolation of the desired fragment, and ligation. For constructions utilizing pLR9, only the *Cla*I-*Pvu*II portion of pBR322 was present in the final construct.

[b] Construction of these plasmids has been described elsewhere (27).

[c] The coordinate of the most upstream nucleotide of the *glnA* promoter region is indicated. DNA containing the restriction site indicated abuts the deletion endpoint.

because *glnG* is transcribed from the weak *glnL* promoter. The maximal expression from the *glnL* promoter, which is negatively controlled by NR_I, is less than that from *glnAp2* during nitrogen-limited growth (21).

We have previously shown that progressive deletion of the upstream high-affinity NR_I-binding sites either diminishes expression initiated from *glnAp2* or increases the concentration of NR_I required for transcription or both (27). These results were obtained by using recombinant *glnA* plasmids in which the upstream boundary of the *glnA* region was known but an uncharacterized portion of pBR322 remained. These deletion plasmids were reconstructed so that all contained a defined region of pBR322, the entire RNA polymerase-binding site for *glnAp2*, DNA encoding the ribosome-binding site and the structural gene for *glnA*, and the first 66 bases of the *glnL* structural gene. The plasmids do not contain the *glnL* or *glnG* gene (Fig. 2). The results from assay of glutamine synthetase activity from cells with the reconstructed plasmids confirmed our previous results (Table 6). The expression of *glnA* was optimal when high-affinity NR_I-binding sites 1 and 2 were present (plasmid pLR146; Table 6): a low concentration of NR_I was as effective as a high concentration. In cells with plasmid pBM12, which has a deletion of the distal high-affinity NR_I-binding site 1, NR_I at a high concentration, but not at a low concentration, could fully stimulate the formation of glutamine synthetase (Table 6). NR_I at a high concentration was 50% effective in activating *glnA* from a plasmid without site 1 and with only a partial site 2 but was only 9 to 20% effective from plasmids without

TABLE 2. Plasmids with variation of the distance between the RNA polymerase-binding site and high-affinity NR_I-binding sites 1 and 2

Plasmid	Ancestral plasmid[a]	Distance[b] (bp)	NR_I-binding site(s)		Reference or derivation[c]
			From insert	From vector	
pLR81	pTH814	32	1, 2	5	27
pLR121	pTH814	75	1–4	5	Inserted 8-bp *Kpn*I linker into *Xho*I site (K) of pTH814
pLR148	pLR12	46	1, 2	4, 5	Inserted 450-bp *Dra*III-*Asp*718 fragment (T4) of pLR1[d] into *Kpn*I site (T4) of pLR145
pLR149	pLR12	50	1, 2	4, 5	Inserted 450-bp *Dra*III-*Asp*718 fragment (T4) of pLR1[d] into *Asp*718 site (K) of pLR145
pLR150	pTH814	79	1–4	5	Filled in *Asp*718 site (K) of pLR121
pLR151	pTH814	71	1–4	5	Blunted *Kpn*I site (T4) of pLR121
pLR152	pLR7	60	1–4	5	Inserted 480-bp *Xho*I-*Sma*I fragment (K) of pTH814[e] into *Asp*718 site (K) of pBM8
pLR153	pLR7	18	1, 2	5	Inserted 450-bp *Dra*III-*Asp*718 fragment (T4) of pLR1[d] into *Kpn*I site (T4) of pBM8
pLR154	pTH814	29	1, 2	5	Inserted 450-bp *Dra*III-*Asp*718 fragment (T4) of pLR1[d] into large fragment of pTH814 cut with *Xho*I-*Pvu*II (K); *Xho*I site recreated; essentially a deletion of sites 3 and 4
pLR155	pLR9	41	1, 2	4, 5	Inserted 450-bp *Dra*III-*Asp*718 fragment (T4) of pLR1[d] into large fragment of pLR9 cut with *Pvu*II-*Asp*718 (K); essentially a deletion of site 3
pLR156	pLR7	24	1, 2	5	Inserted 450-bp *Xho*I-*Sma*I fragment (K) of pLR76[f] into *Kpn*I site (T4) of pBM8
pLR164	pTH814	33	1, 2	5	Filled in *Xho*I site (K) of pLR154
pTH814	pTH814	63	1–4	5	27; inserted 8-bp *Xho*I linker into *Fsp*I site of pgln6

[a] The plasmid described in the first column was ultimately derived from these ancestral plasmids. The upstream endpoint of the wild-type *glnA* promoter region remaining can be determined by referring to Table 1.
[b] Distance between the downstream edge of the proximal high-affinity NR_I-binding site and the upstream edge of the RNA polymerase-binding site.
[c] (K), The staggered end left after cleavage by a restriction endonuclease was filled in with the Klenow fragment of DNA polymerase I from *E. coli*; (T4), after enzymatic cleavage, the 3′ staggered end was blunted or the 5′ staggered end was filled in with DNA polymerase from phage T4. A detailed restriction map of the *glnA* promoter region is given in reference 27.
[d] The insert contains DNA from −92 to −530 of the *glnA* promoter region plus 7 bp of a *Kpn*I (*Asp*718) linker at the promoter-distal end of the insert.
[e] The insert contains DNA from −61 to −530 of the *glnA* promoter region plus 6 bp of an *Xho*I linker at the promoter-proximal end of the insert.
[f] The insert contains DNA from −92 to −530 of the *glnA* promoter region plus 6 bp of an *Xho*I linker at the promoter-proximal end of the insert. Construction of pLR76 is described in reference 27.

sites 1 and 2, regardless of the presence of the low-affinity site 3, 4, or 5 (Table 6). Activation by NR_I at a low concentration apparently requires at least one high-affinity NR_I-binding site.

Expression of *glnA* as a function of the distance between the binding site for RNA polymerase and the two high-affinity binding sites for NR_I. To construct a series of plasmids with various distances between the binding site for RNA poly-

TABLE 3. Plasmids with one high-affinity NR_I-binding site from the *glnL* promoter

Plasmid[a]	Parent[b]	Distance[c] (bp)	Derivation[d]
pBM4	pTH814	48	Inserted 158-bp *Hin*dIII-*Hin*PI fragment (K) of pgln92[e] into *Xho*I site of pBM1 (see Table 1)
pLR94	pLR8	24	Inserted 139-bp *Hin*dIII-*Alu*I fragment (K) of pgln92[f] into the *Asp*718 site (K) of pLR88; a unique *Asp*718 site is created between the binding sites for NR_I and RNA polymerase
pLR96	pLR8	28	Filled in *Asp*718 site (K) of pLR94
pLR98	pLR8	20	Blunted *Kpn*I site (T4) of pLR94
pLR101	pLR8	36	Inserted 8-bp *Hin*dIII linker into *Asp*718 site (K) of pLR94
pLR102	pLR8	38	Inserted 10-bp *Hin*dIII linker into *Asp*718 site (K) of pLR94
pLR103	pLR8	40	Inserted 12-bp *Hin*dIII linker into *Asp*718 site (K) of pLR94
pLR133	pLR8	50	Inserted 8-bp *Xho*I linker into *Hin*dIII site (K) of pLR102
pLR134	pLR8	54	Filled in *Xho*I site (K) of pLR133
pLR135	pLR8	52	Inserted 8-bp *Xho*I linker into *Hin*dIII site (K) of pLR103
pLR137	pLR8	56	Filled in *Xho*I site (K) of pLR135
pLR139	pLR8	66	Inserted 12-bp *Hin*dIII linker into *Xho*I site (K) of pLR133
pLR143	pLR8	60	Inserted 6-bp *Sma*I linker into *Xho*I site (K) of pLR133
pLR165	pLR8	70	Filled in *Hin*dIII site (K) of pLR139

[a] All contain the low-affinity NR_I-binding site 5 but not sites 1 through 4. NR_I does not detectably bind to site 5 in whole cells (31).
[b] See footnote *a*, Table 2.
[c] Distance between the downstream edge of the high-affinity NR_I-binding site and the upstream edge of the RNA polymerase-binding site.
[d] (K), The staggered end left after cleavage by a restriction endonuclease was filled in with the Klenow fragment of DNA polymerase I from *E. coli*; (T4), after enzymatic cleavage, the 3′ staggered end was blunted or the 5′ staggered end was filled in with DNA polymerase from phage T4.
[e] This fragment from pgln92 contains DNA from −31 to +121 of the *glnL* promoter region plus part of the *Hin*dIII linker; the NR_I-binding site is from −6 to +11. The sequence and coordinates are given in reference 32. Only the −10 half of the *glnL* promoter remains and would direct transcription away from *glnAp2* if it were active.
[f] As in footnote *e*, but the DNA from the *glnL* promoter region is from −12 to +121 plus part of the *Hin*dIII linker.

TABLE 4. Plasmids with DNA added between the RNA polymerase-binding site and a partial high-affinity NR_I-binding site

Plasmid[a]	No. of bases added	Derivation[b]
pLR170	4	Blunted 3′ overhang of *Kpn*I site of pLR171 (T4) (listed below); *Not*I site created
pLR171	8	Inserted 8-bp *Kpn*I linker into *Fsp*I site of pLR169 (see Table 1) between coordinates −60 and −61
pLR172	10	Inserted 10-bp *Hin*dIII linker into *Fsp*I site of pLR169 (see Table 1) between coordinates −60 and −61
pLR173	14	Filled in *Hin*dIII site (K) of pLR172; *Nhe*I site created
pLR174	20	Inserted 8-bp *Hin*dIII linker into *Asp*718 site (K) of pLR171
pLR177	24	Filled in *Hin*dIII site (K) of pLR174
pLR180	16	Inserted 8-bp *Kpn*I linker into *Not*I site (K) of pLR170
pLR181	26	Inserted 8-bp *Kpn*I linker into *Nhe*I site (K) of pLR173

[a] All are derivatives of pLR169 (see Table 1), and all contain NR_I-binding sites 3 through 5 and half of the high-affinity site 2.
[b] (K), The staggered end left after cleavage by a restriction endonuclease was filled in with the Klenow fragment of DNA polymerase I from *E. coli*; (T4), after enzymatic cleavage, the 3′ staggered end was blunted or the 5′ staggered end was filled in with DNA polymerase from phage T4.

merase and the two high-affinity NR_I-binding sites, DNA containing the high-affinity sites 1 and 2 of the *glnA* promoter region was inserted into a unique restriction endonuclease site 7 to 77 bases upstream of the binding site for RNA polymerase (Fig. 2A and B). The constructions generally resulted in the creation of a unique restriction site between the two regions of interest; the insertion of synthetic oligonucleotides into these unique sites permitted further alter-

TABLE 5. Plasmids with variations of the distance between the two high-affinity NR_I-binding sites

Plasmid[a]	No. of bases added	Derivation[b]
pBM13	4	Inserted 139-bp *Alu*I-*Hin*dIII fragment (K) of pgln92[c] into *Asp*718 site (K) of pBM12; a unique *Asp*718 site is created between the two NR_I-binding sites
pLR108	8	Filled in *Asp*718 site (K) of pBM13
pLR109	10	Inserted 10-bp *Hin*dIII linker into *Kpn*I site (T4) of pBM13
pLR111	16	Inserted 8-bp *Hin*dIII linker into *Asp*718 site (K) of pBM13
pLR112	18	Inserted 10-bp *Hin*dIII linker into *Asp*718 site (K) of pBM13
pLR114	14	Filled in *Hin*dIII site (K) of pLR109
pLR115	22	Filled in *Hin*dIII site (K) of pLR112

[a] All are derivatives of pBM12 (see Table 1) and contain NR_I-binding sites 2 through 5.
[b] (K), The staggered end left after cleavage by a restriction endonuclease was filled in with the Klenow fragment of DNA polymerase I from *E. coli*; (T4), after enzymatic cleavage, the 3′ staggered end was blunted or the 5′ staggered end was filled in with DNA polymerase from phage T4.
[c] This fragment from pgln92 contains DNA from −31 to +121 of the *glnL* promoter region plus part of the *Hin*dIII linker; the NR_I-binding site is from −6 to +11. The sequence and coordinates are given in reference 32. Only the −10 half of the *glnL* promoter remains and would direct transcription away from *glnAp2* if it were active.

TABLE 6. Activation of *glnA* from plasmids with deletions of the NR_I-binding sites

Plasmid	Upstream boundary[a]	NR_I site present[b]					% of full activation[c]	
		1	2	3	4	5	Low NR_I	High NR_I
pLR146	−540	+	+	+	+	+	94	100[d]
pBM12	−122	−	+	+	+	+	42	81
pLR169	−109	−	1/2	+	+	+	15	43
pLR157	−94	−	−	+	+	+	5	20
pLR145	−80	−	−	−	+	+	2	9
pLR88	−55	−	−	−	−	+	4	19

[a] The plasmids are identical except for the extent of the region upstream of the start site of transcription from *glnAp2* (see Table 1 for details of construction). The upstream boundary of the *glnA* region of each plasmid abuts the unique *Pvu*II site of pBR322.
[b] See Fig. 1 for NR_I-binding sites.
[c] See Methods and Materials for a description of the strains used to obtain these values and the corrections applied.
[d] This value was defined as 100%.

ations. The distance between the two regions of interest was varied by a number of turns of the DNA helix so that trends would be obvious and conclusions would not be based on results for a few plasmids.

The wild-type distance between the downstream boundary of the proximal high-affinity NR_I-binding site and the upstream edge of the RNA polymerase-binding site is 55 bp. NR_I stimulated the synthesis of glutamine synthetase from a plasmid with the wild-type *glnA* promoter region as well as from plasmids in which this distance was 29, 33, 40, 46, 60, 63, 67, 71, 75, or 79 bp. Glutamine synthetase activity was only 25% of optimal in cells with plasmid pLR156, in which the two high-affinity NR_I-binding sites were within 24 bp of the RNA polymerase-binding site, which places binding site 1 at the position occupied by site 2 for a wild-type *glnA* gene. NR_I-phosphate in low or high concentration stimulated glutamine synthetase formation from *glnA* of pLR156 to the same extent (Fig. 3). When the NR_I-binding sites were 18 bp from RNA polymerase, NR_I-phosphate in low concentration did not stimulate the synthesis of glutamine synthetase, whereas NR_I-phosphate in high concentration stimulated transcription within the range of values measured in cells containing plasmids without NR_I-binding sites.

Cells with plasmids pLR81 and pLR149, in which the downstream boundaries of the proximal high-affinity NR_I-binding sites were 32 and 50 bp, respectively, from the RNA polymerase-binding site, had significantly lower glutamine synthetase activity than did cells with plasmid pLR146, which has the wild-type *glnA* promoter region (Fig. 3). The results from cells with plasmids pLR81 and pLR149 were not representative of a trend.

From these results, we draw two major conclusions: NR_I-phosphate must be bound to sites that are a minimal distance from RNA polymerase to stimulate *glnA* transcription, and there is no periodic variation of *glnA* expression when the high-affinity binding sites are displaced half or whole integral turns of the DNA helix relative to the binding site for RNA polymerase. Plasmids pLR148 and pLR155 lack site 3, which is occupied in whole cells by NR_I, but have sites 1, 2, 4, and 5. Nonetheless, cells with these plasmids had a wild-type level of glutamine synthetase, which suggests that NR_I bound to site 3 does not significantly affect transcription from *glnAp2* in the presence of the two high-affinity NR_I-binding sites.

Activation of *glnAp2* by NR_I bound to one high-affinity binding site. We constructed a series of recombinant plas-

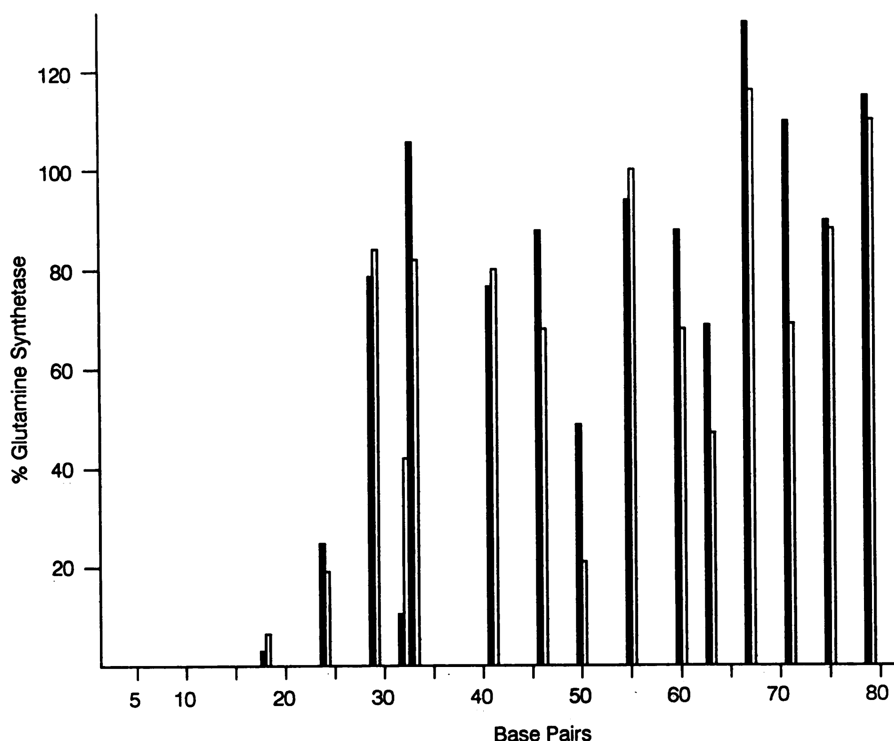

FIG. 3. Glutamine synthetase activity from cells with alterations in the distance between the RNA polymerase-binding site and the two high-affinity NR$_I$-binding sites. The coordinates refer to the distance between the downstream boundary of the proximal high-affinity NR$_I$-binding site and the upstream boundary of the RNA polymerase-binding site. The wild-type spacing is 55 bp (see Fig. 1). The plasmids were constructed by inserting DNA containing NR$_I$-binding sites 1 and 2 at the boundaries of deletions upstream of the RNA polymerase-binding site. The spacing between the two high-affinity NR$_I$-binding sites is the wild-type spacing. Details of the constructions are given in Materials and Methods, Tables 1 and 2, and Fig. 2. The values of glutamine synthetase were compared with those obtained from cells of YMC10 with plasmid pLR146. Bars indicate glutamine synthetase activity resulting from transcription by low (■) and high (□) concentrations of NR$_I$, respectively. The values presented are averages of at least two independent determinations. The average standard deviations of these values from their means were 8.9% for cells with a low level of NR$_I$ and 9.4% for cells with a high level of NR$_I$.

mids in which DNA containing a single high-affinity NR$_I$-binding site from the *glnL* promoter region was ligated upstream of the RNA polymerase binding site of *glnAp2* (Fig. 2A). In plasmids of this series, sites 1 through 4 of the *glnA* promoter region have been deleted. We inserted DNA with the NR$_I$-binding site from the *glnL* promoter, which is highly homologous to the high-affinity NR$_I$-binding sites upstream from *glnAp2* (Table 7), for ease of construction; furthermore, this DNA does not have low-affinity NR$_I$-binding sites to potentially complicate interpretations. Glutamine synthetase activity was never as high in cells with these plasmids as in cells containing plasmid pBM12, which has only one high-affinity NR$_I$-binding site, site 2, but also has site 3 from the *glnA* promoter region (Table 6). It is possible that the low-affinity NR$_I$-binding site participates in the stimulation of transcription when there is only one high-affinity NR$_I$-binding site: this point will be discussed in a later section.

In cells with plasmids in which the *glnL* NR$_I$-binding site was 55 bp or more from the binding site for RNA polymerase, a low or high concentration of NR$_I$ induced the synthesis of glutamine synthetase to a level higher than that observed in cells containing plasmids without NR$_I$-binding sites (Fig. 4). A moderate stimulation of transcription by a low level of NR$_I$ was apparent even when the NR$_I$-binding site was as close as 36 bp from the RNA polymerase-binding site. However, when the single high-affinity NR$_I$-binding site was closer than the wild-type site 2, which is 55 bp from the RNA polymerase-binding site, NR$_I$ in high concentration did not stimulate the formation of glutamine synthetase above the level observed in cells bearing plasmids without high-affinity NR$_I$-binding sites (Fig. 4).

We conclude that NR$_I$ bound to a single high-affinity site must be a minimal distance from RNA polymerase to effectively activate transcription and that when the distance is greater than this minimum, activation is not dependent on an exact spatial relationship between one binding site for NR$_I$ and the binding site for RNA polymerase. Because the results presented in Fig. 3 and 4 led to essentially the same conclusions, these conclusions are not based on one particular type of plasmid construction with one particular NR$_I$-binding site or on the presence of the low-affinity NR$_I$-binding sites 3 and 4 on the plasmids. The data presented in Fig. 4 were obtained from cells with plasmids that do not have the low-affinity sites 3 and 4; although site 5 is present

TABLE 7. Sequences of NR$_I$-binding sites

Gene	Site	Sequence
glnAp2	1	TGCACCaacaTGGTGCT
glnAp2	2	AGCACTatatTGGTGCA
glnLp		TGCACTaaaaTGGTGCA
glnAp2	3	ATTCACatcgTGGTGCA
Consensus		TGCAC$^{C}_{T}$NNNNTGGTGCA

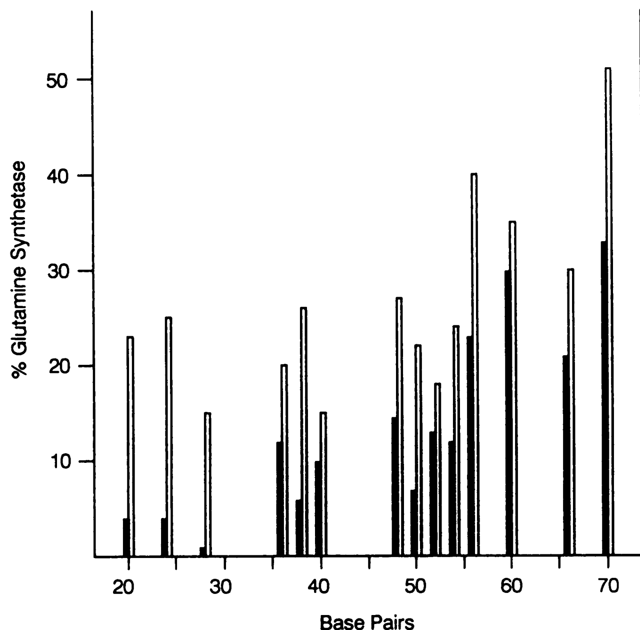

FIG. 4. Influence of variation of the distance between the RNA polymerase-binding site and a single high-affinity binding site for NR_I from the *glnL* promoter on glutamine synthetase activity. Coordinates refer to the distance between the downstream boundary of the high-affinity NR_I-binding site and the upstream edge of the RNA polymerase-binding site. Details of the constructions are given in Table 3. All of these plasmids contain the low-affinity site 5 but lack sites 3 and 4. Bars indicate the glutamine synthetase activity resulting from transcription by low (■) and high (□) concentrations of NR_I, respectively. Glutamine synthetase activity is compared with that observed from cells of strain YMC10 with plasmid pLR146, which has two high-affinity NR_I-binding sites. This external standard is used so that all data presented in this paper can be directly compared. NR_I-dependent stimulation of *glnAp2* without the upstream sites would result in about 15% of the optimal activity from cells of strain YMC10 with plasmid pLR146 and is not subtracted from the data presented in the figure. The values presented are averages of two independent determinations. The average standard deviations of the values from the means were 20.9% for cells with a low level of NR_I and 8.5% for cells with a high level of NR_I.

in this set of plasmids, NR_I does not occupy site 5 in whole cells (31).

Varying the position of a partial NR_I-binding site. NR_I-phosphate can still activate the expression of *glnA* from a partial binding site 2 (plasmid pLR169; Table 6). We varied the distance between the partial NR_I-binding site and the RNA polymerase-binding site by inserting synthetic oligonucleotides between the two sites (Fig. 2C). Periodic activation, dependent on the location of the partial NR_I-binding site, was evident for *glnA* expression by a high level of NR_I (Fig. 5). The results are more dramatic if it is remembered that one type of background activity, expression from *glnAp2* without NR_I-binding sites (9 to 20%; Table 6), has not been subtracted; in other words, this periodic activation was virtually an all-or-none phenomenon. A low level of NR_I did not stimulate the transcription of glutamine synthetase significantly in cells with this set of plasmids (L. Reitzer, unpublished observation).

Variation of the distance between two high-affinity NR_I-binding sites. DNA containing the high-affinity NR_I-binding site from the *glnL* promoter was inserted into pBM12 upstream of the intact high-affinity binding site 2 (Fig. 2D). We inserted oligonucleotides into a restriction endonuclease site between the two high-affinity NR_I-binding sites to further vary the distance. When the distance between the two NR_I-binding sites was greater than the wild-type distance by integral turns of the DNA helix, *glnA* transcription was optimal compared with when the distance was increased by half integral turns of the DNA helix. The same results were observed whether *glnA* was activated by a low or high level of NR_I (Fig. 6). These results show that the NR_I-binding site from the *glnL* promoter can completely replace the high-affinity site 1 of the *glnA* promoter. These results also show that the spacing between two high-affinity NR_I-binding sites is important for optimal activation.

DISCUSSION

We confirmed our earlier observations that optimal transcription from *glnAp2* requires the binding of NR_I-phosphate to high-affinity NR_I-binding sites 1 and 2. Without these sites, a high intracellular concentration of NR_I still resulted in about 15% of the maximal transcription from *glnAp2*, irrespective of the presence of the low-affinity binding site 3, 4, or 5. The two high-affinity NR_I-binding sites not only increase the rate of glutamine synthetase formation in cells with a high intracellular concentration of NR_I but also make it possible to achieve this high rate in cells containing NR_I in low concentration (Table 6; 20, 27).

Ptashne has reviewed mechanisms of transcriptional activation by proteins bound to DNA either distant or near the start site of transcription (24). There are four general mechanisms for the action of these positive regulators: RNA polymerase-activator interactions, which may occur across large intervening stretches of DNA; a conformational change in DNA that is stabilized or induced by the activator and is transmitted to the site of regulation; the binding of a regulator to one site, followed by its movement or sliding along the DNA to a regulatory site; and binding of a regulatory protein to one site that allows the cooperative binding of possibly multiple molecules of the regulator until the site of regulation is reached. We will argue that our observations support the first model of a direct interaction of an activator with RNA polymerase.

The results from experiments with whole cells and with purified components in a completely defined system have demonstrated that the activation of transcription from the high-affinity NR_I-binding sites is fully effective even when the sites are more than 1,000 bp from the RNA polymerase-binding site (20, 27). Fifty-five base pairs separate the downstream boundary of the proximal high-affinity NR_I-binding site (site 2) from the upstream edge of the RNA polymerase-binding site for the wild-type *glnA* gene. The data presented in Fig. 3 and 4, despite differences in details, show that at least about 30 bp must separate RNA polymerase from NR_I-phosphate bound to a single site or to two high-affinity upstream sites for the stimulation of transcription from *glnAp2*. Except for this restriction, the exact spacing between the binding sites for RNA polymerase and the activator does not affect transcription from *glnAp2*. This requirement for a minimal distance between the binding site for RNA polymerase and the binding site or sites for NR_I is most easily accommodated by the model that a long-range NR_I-RNA polymerase interaction stimulates transcription from *glnAp2*.

The direct-contact hypothesis is strongly supported by the observation that for activation by NR_I-phosphate from a partial binding site, only integral turns of DNA can be added

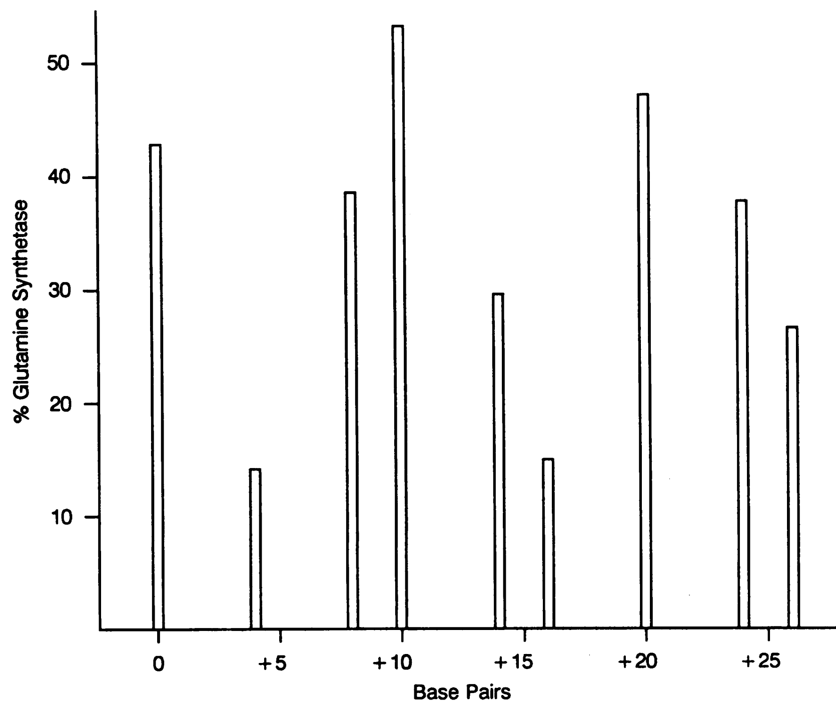

FIG. 5. Effect on glutamine synthetase activity of increasing the distance between a partial NR_I-binding site 2 and the RNA polymerase-binding site. Coordinates refer to the number of base pairs added between the binding site for RNA polymerase and the partial NR_I-binding site. Plasmids of this series were derived from plasmid pLR14, which contains only half of NR_I-binding site 2 but all of the low-affinity sites 3 through 5. The distance between NR_I-binding site 2 and the RNA polymerase-binding site was varied by the insertion of oligonucleotides between the low-affinity NR_I-binding sites 4 and 5; the relative position of the partial site 2 to sites 3 and 4 is constant in these plasmids. Bars indicate the glutamine synthetase activity resulting from transcription by a high concentration of NR_I, respectively; 100% glutamine synthetase activity is the value observed from cells of strain YMC10 containing plasmid pLR146, which has two high-affinity NR_I-binding sites. This external standard is used so that all data presented in this paper can be directly compared. NR_I-dependent stimulation of *glnAp2* without the upstream sites would result in about 15% of the optimal activity from cells of strain YMC10 with plasmid pLR146 and is not subtracted from the data presented in the figure. The values presented are averages of at least three independent determinations. The average standard deviation of these values from their means was 8.3%.

between the binding sites for RNA polymerase and NR_I (Fig. 5). The fact that NR_I-phosphate binds weakly to a partial binding site can be inferred from the difference in glutamine synthetase activity from cells with plasmid pLR169 which contains the partial NR_I binding-site 2, compared with cells with plasmid pBM12, which has the entire site 2 (Table 6). The phase-dependent activation by NR_I from the partial binding site virtually eliminates the possibility that either NR_I-phosphate or RNA polymerase slides along the DNA helix.

The results of studies on the binding of purified σ^{54}-RNA polymerase and NR_I to DNA are consistent with the hypothesis that NR_I-phosphate contacts RNA polymerase. DNA-bound σ^{54}-RNA polymerase facilitates the binding of NR_I-phosphate to sites 1 and 2 twofold, but not the binding of unmodified NR_I (see Fig. 1D of reference 20). A twofold enhancement is probably an underestimate because the extent of phosphorylation of NR_I with purified components tends to be low (19). This apparent cooperativity is consistent with a physical interaction between these heterologous proteins. A physical interaction between another activator, the cyclic AMP receptor protein, and RNA polymerase has been proposed, in part on the basis of similar evidence (29).

NR_I-phosphate stimulates the conversion of a closed promoter complex to an open promoter complex in a reconstituted system (18, 22). The majority of fully activated *glnAp2* promoters are bound by σ^{54}-RNA polymerase in a closed, rather than an open, promoter complex in intact cells. Only after the addition of rifampin, an inhibitor of the elongation of transcription, was the open promoter complex detectable in intact cells (31). This result, together with the results presented here, implies that the interaction of NR_I-phosphate with RNA polymerase is the rate-limiting step for the formation of the open promoter complex and for the initiation of transcription from *glnAp2*.

Other complex aspects of the control of transcription from the *glnAp2* promoter, which are discussed below, can also be reconciled with a model of regulation that involves a potentially long-range interaction between NR_I-phosphate and RNA polymerase. These other results, in aggregate, are difficult to reconcile with one single alternative mechanism of activation by NR_I. Possibly the most surprising result of this work is that in contrast to activation from a weak NR_I-binding site, NR_I-phosphate tightly bound to an intact high-affinity site or sites can activate from either side of the DNA helix (Fig. 3 and 4). We are not aware of an analogous situation in any other system, procaryotic or eucaryotic. For the purpose of illustrating how this result can be reconciled with the contact hypothesis, assume that when NR_I-phosphate is bound to one side of the DNA helix, a productive contact with RNA polymerase is faster than when NR_I-phosphate is bound on the other side of the DNA. Assume also that the average time of occupancy of NR_I is sufficiently long to allow a contact with RNA polymerase when NR_I is bound to an intact high-affinity site on either side of the DNA helix. However, the shorter average time of occupancy

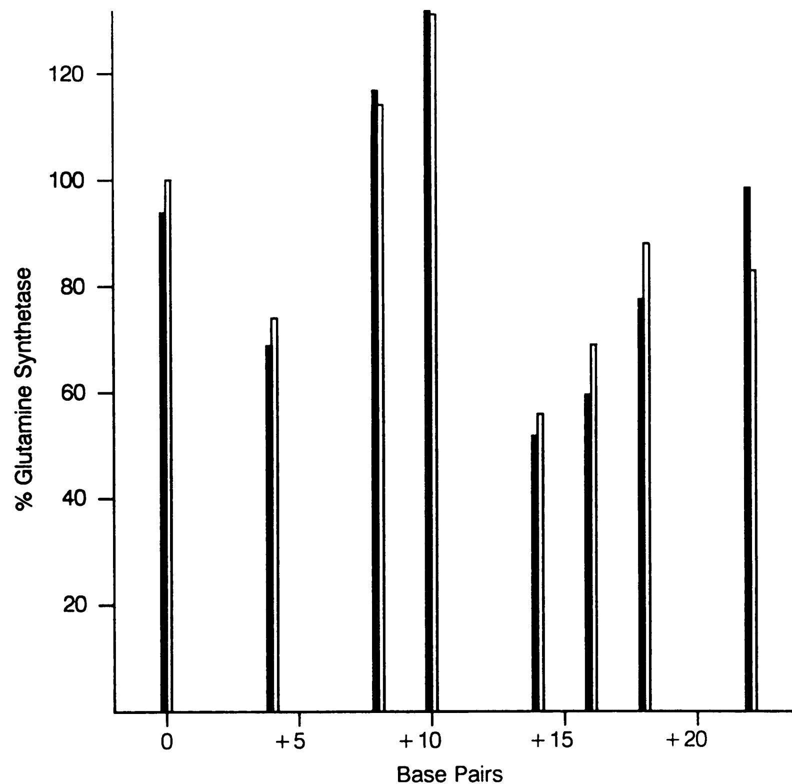

FIG. 6. Effect of variation of the distance between two high-affinity NR_I-binding sites on glutamine synthetase activity. DNA containing the high-affinity NR_I-binding site from the *glnL* promoter was inserted into plasmid pLR15 upstream of site 2. All plasmids of this series contain the high-affinity site 2 and the low-affinity sites 3 through 5. Other details of the constructions are given in Table 5. Coordinates refer to the number of base pairs added between the two high-affinity NR_I-binding sites compared with the wild-type distance between sites 1 and 2. Bars indicate glutamine synthetase activity observed from cells with low (■) and high (□) concentrations of NR_I, respectively, compared with that observed in strain YMC10 with plasmid pLR146, which has two high-affinity NR_I-binding sites. This external standard is used so that all data presented in this paper can be directly compared. NR_I-dependent stimulation of *glnAp2* without the upstream sites would result in about 15% of the optimal activity from cells of strain YMC10 with plasmid pLR146 and is not subtracted from the data presented in the figure. The plasmid used to obtain the data for coordinate zero was pLR146, and the data were added to the figure only as a reference. The values presented are averages of two independent determinations. The average standard deviations of these values from their means were 6.2% for cells with a low level of NR_I and 7.6% for cells with high level of NR_I.

when NR_I is bound to a weakened binding site may allow a contact with RNA polymerase from only one side of the DNA helix.

Two plasmids with two high-affinity NR_I-binding sites were constructed in which the distal site should have been sufficiently far from the RNA polymerase-binding site for NR_I-dependent stimulation of transcription from *glnAp2*, but the proximal site was too close. There was four times less glutamine synthetase in cells with plasmid pLR156, which has two high-affinity NR_I-binding sites 55 and 24 bp from the RNA polymerase-binding site, than in cells with plasmid pBM12, which has one high-affinity site 55 bp from RNA polymerase-binding site (Fig. 3 and Table 6). Furthermore, nitrogen limitation could not induce the formation of glutamine synthetase in cells with plasmid pLR153, which has high-affinity sites 49 and 18 bp from the RNA polymerase-binding site (Fig. 3). In plasmids pLR153 and pLR156, the wild-type distance between the high-affinity NR_I-binding sites was maintained. We interpret these results to imply that NR_I-phosphate bound to the proximal site might diminish the flexibility of the DNA required for the interaction of RNA polymerase with NR_I-phosphate bound to a distal site. Brent and Ptashne have observed a similar type of interference (3).

Adding half integral turns of DNA between two high-affinity NR_I-binding sites resulted in a lower level of *glnA* transcription (Fig. 6). These results show that *glnA* expression is optimal when two high-affinity sites are on the same face of the DNA helix because all of the contacts made by NR_I at sites 1 and 2 are on the same face (9, 14, 20, 31). NR_I bound to an inappropriately spaced high-affinity site actually interferes with activation stimulated from a neighboring site because there is more glutamine synthetase activity from cells with plasmid pBM12, containing only sites 2 through 5, than from cells with plasmids such as pBM13, pLR111, and pLR114, containing sites 2 through 5 plus an improperly spaced high-affinity site. One possible explanation for this interference is that NR_I-phosphate bound to one site might prevent DNA from bending away from the bound NR_I and hinder a second molecule of NR_I-phosphate bound on the opposite face of the DNA from contacting RNA polymerase. According to this hypothesis, when both NR_I-binding sites are on the same side of the DNA, the directional restriction would not affect contacts between NR_I-phosphate and RNA polymerase. Other complex possibilities might be imagined, but potential explanations that invoke cooperative interactions between NR_I molecules bound to adjacent high-affinity sites would not be supported by experimental evidence (20).

Giniger and Ptashne observed that even though binding of the GAL4 protein of *Saccharomyces cerevisiae* to adjacent

high-affinity binding sites was not cooperative, binding to a low-affinity site was facilitated by the occupancy of an adjacent high-affinity site (8). Cooperative interactions between molecules of NR_I-phosphate bound to adjacent low-affinity sites could explain why NR_I-phosphate bound to the partial site 2 can still activate transcription (plasmid pLR169; Table 6). This result was unexpected because the promoter-proximal half of site 2 is virtually identical in nucleotide sequence to the promoter-proximal half of site 3 (Table 7), from which NR_I could not stimulate *glnA* transcription (pLR157; Table 6). Nonetheless, NR_I at a high intracellular concentration occupies site 3 in vivo (31), and the contacts made by NR_I at site 3 are on the same face of the helix as those at sites 1 and 2 (9, 14, 20, 31). These observations are consistent with the hypothesis that NR_I-phosphate bound to site 3 might strengthen the binding of NR_I-phosphate to the partial site 2 and might facilitate activation from *glnAp2*. An auxiliary role of binding site 3 may also explain the higher level of *glnA* expression from plasmid pBM12 (Table 6), which contains only one high-affinity site (site 2), than from plasmids with the single high-affinity NR_I-binding site from the *glnL* promoter (Fig. 4): the latter set of plasmids lacks binding site 3.

A contact between RNA polymerase and an activator that involves DNA loop formation has also been proposed for activation in two other procaryotic systems. The activation of the *nifH* promoter of *Klebsiella pneumoniae* and of other *nif* promoters requires the product of the *nifA* gene, instead of NR_I, and σ^{54}-RNA polymerase. Deletion of the presumed binding site for the *nifA* product, located 130 bp upstream from the transcription start site, reduces the transcription of *nifH* about 30-fold (5). On low-copy plasmids, but not on high-copy plasmids, moving the *nifA* product-binding site 5 or 15 bp further upstream greatly diminishes the expression of *nifH*, but moving the site 11 bp further upstream does not affect expression (4). Placement of the putative *nifA* product-binding site 21 bp (only two turns of the helix) and 1,000 bp further upstream decreases activation 80 and 90%, respectively (4, 5). This spatial requirement suggested that the *nifA* product contacts RNA polymerase, although the conclusion is based on a positive result from cells with one plasmid and only with a low-copy plasmid. Activation of the *ompC* gene by the *ompR* gene product is similar to activation of *nifH* by the *nifA* gene product: only integral turns of DNA can be inserted between binding sites for the *ompR* protein and RNA polymerase. Furthermore, the effectiveness of the binding site for the *ompR* protein diminishes rapidly with increasing distance from the RNA polymerase-binding site (15). In contrast to activation stimulated by the products of the *nifA* and *ompR* genes, moving the activator-binding sites 1,400 bp upstream from *glnAp2* does not affect activation. It is in fact difficult to reconcile the sharp diminution of transcriptional activation by the *nifA* and *ompR* gene products with increasing distance simply by postulating a contact between the activator and RNA polymerase.

In summary, we propose the following sequence of events leading to the initiation of transcription from *glnAp2*. A specific protein kinase phosphorylates NR_I when there is a shortage of assimilable nitrogen in the medium. Maximal transcription from *glnAp2* requires the binding of NR_I-phosphate to sites on the DNA that must be at least about 30 bp from the upstream boundary of the σ^{54}-RNA polymerase-binding site. The mechanism by which NR_I-phosphate bound to upstream sites stimulates the transcription of *glnAp2* is most easily accommodated by postulating that NR_I-phosphate contacts RNA polymerase. This contact results in the formation of an open promoter complex and is the rate-limiting step in the initiation of transcription of the *glnA* gene. Other complex aspects of the regulation of NR_I-dependent transcription of *glnA* that distinguish it from other systems can also be reconciled with the hypothesis of an interaction between NR_I-phosphate and RNA polymerase.

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