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Activation of Transcription Initiation from the *nac* Promoter of *Klebsiella aerogenes*

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The *nac* gene of *Klebsiella aerogenes* encodes a bifunctional transcription factor that activates or represses the expression of several operons under conditions of nitrogen limitation. In experiments with purified components, transcription from the *nac* promoter was initiated by σ^{54} RNA polymerase and was activated by the phosphorylated form of nitrogen regulator I (NRI) (NtrC). The activation of the *nac* promoter required a higher concentration of NRI~P than did the activation of the *Escherichia coli* $glnAp_2$ promoter, and both the promoter and upstream enhancer element contributed to this difference. The *nac* promoter had a lower affinity for σ^{54} RNA polymerase than did $glnAp_2$, and uninitiated competitor-resistant transcription complexes formed at the *nac* promoter decayed to competitor-sensitive complexes at a greater rate than did similar complexes formed at the $glnAp_2$ promoter. The *nac* enhancer, consisting of a single high-affinity NRI-binding site and an adjacent site with low affinity for NRI, was less efficient in stimulating transcription than was the *glnA* enhancer, which consists of two adjacent high-affinity NRI-binding sites. When these binding sites were exchanged, transcription from the *nac* promoter was increased and transcription from the $glnAp_2$ promoter was decreased at low concentrations of NRI~P. Another indication of the difference in the efficiency of these enhancers is that although activation of a *nac* promoter construct containing the *glnA* enhancer was relatively insensitive to subtle alterations in the position of these sites relative to the position of the promoter, activation of the natural *nac* promoter or a *nac* promoter construct containing only a single high-affinity NRI~P binding site was strongly affected by subtle alterations in the position of the NRI~P binding site(s), indicating a face-of-the-helix dependency for activation.

Gram-negative enteric bacteria such as *Escherichia coli*, *Salmonella typhimurium*, and *Klebsiella aerogenes* regulate the expression of *glnA*, encoding glutamine synthetase, and several other genes and operons, collectively known as the Ntr regulon, in response to the availability of the preferred nitrogen source, ammonia (reviewed in references 3, 20–23, and 48). The Ntr genes and operons encode products that permit the use of alternative nitrogen sources when ammonia is absent. Within the Ntr regulon, the *hut* and *put* operons of *K. aerogenes* encode products that permit the utilization of histidine and proline as the sole carbon or nitrogen source (3, 6). The *K. aerogenes hut* and *put* operons are also part of the globally controlled response to carbon starvation (Cer regulon [23]), and transcription of *hut* and *put* can be activated either by carbon starvation (by the cyclic AMP [cAMP]-cAMP response protein [CRP] complex) or by nitrogen starvation (6, 27, 32, 37; reviewed in reference 23).

The expression of *glnA* and the Ntr regulon in response to nitrogen limitation is activated by the phosphorylated form of the *glnG* (*ntrC*) product, nitrogen regulator I (NRI) (30). The response to nitrogen availability is due to the control of the reversible phosphorylation of NRI, accomplished by a signal transduction system consisting of at least three other proteins (reviewed in references 20, 21, and 48). The *glnG* gene is part of the complex *glnALG* operon, and expression of *glnG* is due to transcription from two promoters (34). Transcription from the relatively weak *glnL* promoter provides a low concentration of NRI under conditions of nitrogen excess, and transcription from the nitrogen-regulated $glnAp_2$ promoter, activated by NRI~P, results in a 10-fold-higher concentration of NRI in nitrogen-starved cells (34, 38, 39). Experiments with intact cells indicated that the activation of $glnAp_2$ in response to nitrogen limitation occurs at the low concentration of NRI found in cells at the onset of nitrogen limitation, but that the expression of the *hut* operons requires the higher concentration of NRI that results from the sustained activation of the $glnAp_2$ promoter (34). Indeed, the elevated concentration of NRI~P required for the activation of *hut* expression also results in a decrease in *glnA* expression (7, 47). Thus, the response to nitrogen limitation can be thought of as a temporal cascade, with *glnA* activation representing the initial response to nitrogen limitation and *hut* activation (and a reduction in *glnA* expression) representing a subsequent step to obtain a nitrogen supply when ammonia is absent. The accumulation of a high intracellular concentration of NRI (and its activation by phosphorylation) apparently represents the switching mechanism controlling progression beyond the top (*glnA*) level of the cascade.

Studies with purified components indicated that the $glnAp_2$ promoter is transcribed by RNA polymerase containing σ^{54} instead of the usual σ^{70} and is activated by NRI~P (14, 15, 30; reviewed in references 21 and 48). The $glnAp_2$ control region has two high-affinity NRI-binding sites that are centered 109 and 141 bp upstream from the transcription start site (14). These high-affinity NRI-binding sites are required for the activation of $glnAp_2$ transcription at low concentrations of NRI

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both in vivo and in vitro (31, 40). The pair of high-affinity NRI-binding sites retain the capacity to stimulate $glnAp_2$ transcription at low activator concentrations when moved to new positions far upstream or downstream from the promoter, and in this respect they are analogous to the enhancer sequences of eucaryotic cells (31, 40; reviewed in references 17 and 21–23). Several lines of evidence suggest that NRI~P, bound to its binding sites, interacts with σ^{54} RNA polymerase bound at the promoter in a closed complex by means of a looping-out of the intervening DNA (8, 29, 31, 35, 40, 49, 52, 53; reviewed in references 9, 17, and 22). The interaction of NRI~P and the closed complex results in the formation of the open complex, in which RNA polymerase has melted the DNA strands near the transcriptional start site (34). In the absence of the two high-affinity NRI-binding sites, transcription from $glnAp_2$ can still be activated by NRI~P, but a much higher concentration of this activator is then required (29, 31, 35, 40).

In order to activate transcription, the normally dimeric NRI must become oligomerized, probably to a tetrameric form, and the pair of high-affinity NRI-binding sites upstream from the *glnA* promoter facilitate this process. When the two high-affinity NRI-binding sites from the *glnA* promoter were systematically separated by addition of DNA between them, it was observed that these two sites could act most efficiently only when on the same face of the helix, implying an interaction between the NRI~P dimers bound there (41). The phosphorylation of NRI results in the stimulation of an intrinsic ATPase activity encoded by the central domain of NRI (1, 11, 50). This ATPase activity is necessary (but not sufficient) for the activation of transcription by NRI~P (50). When NRI~P is phosphorylated in solution, the ATPase activity is strongly dependent on the concentration of NRI~P, indicating that an intermolecular interaction must occur (11, 50). Furthermore, the presence of the pair of high-affinity NRI~P-binding sites from the *glnA* promoter greatly stimulates the ATPase activity of NRI~P (1). Whereas unphosphorylated NRI binds to the pair of adjacent binding sites with little cooperativity, NRI~P binds to these same sites with considerable cooperativity (51). Finally, it has been deduced that an oligomeric form of NRI~P is the activating species even when a single NRI~P-binding site is present (36). This conclusion is based on the observation that transcription from a template containing a single NRI-binding site is stimulated by an altered form of NRI~P which has little, if any, ability to bind DNA but can act synergistically with a low concentration of NRI~P able to bind DNA (36).

Transcription of the *hut* operons in response to nitrogen limitation occurs from the same σ^{70}-dependent promoter that is stimulated by the cAMP-CRP complex under condition of carbon limitation, and the *hut* and *put* operons do not contain σ^{54}-dependent promoters (6, 28, 33). Under conditions of nitrogen limitation, the activation of the *hut* and *put* operons and several other Ntr operons requires the product of the *nac* gene (4, 5, 12, 19, 45, 46; reviewed in reference 3). Genetic experiments indicate that the *nac* product is not required for expression from $glnAp_2$ (4) and that the *nac* gene is an Ntr gene under the control of NRI and σ^{54} RNA polymerase (5). The product of *nac* is sufficient for the activation of *hut* transcription in the absence of nitrogen limitation and in the absence of NRI, as indicated by experiments in which the *nac* product was programmed from the isopropylthiogalactopyranoside (IPTG)-inducible *tac* promoter (46). Those experiments eliminated the possibility that NRI~P is directly involved in the activation of *hut* transcription.

The DNA sequence of *nac* indicated that Nac is a member of the LysR family of proteins (45). The other members of this family of proteins are activators (and sometimes also repressors) of σ^{70}-dependent transcription. Sequence analysis of the *nac* regulatory region revealed a sequence with strong similarity to the consensus for σ^{54}-dependent promoters, as well as a sequence with strong similarity to a high-affinity NRI-binding site (45). These findings led to the simple hypotheses that NRI~P activates *nac* expression and that Nac activates *hut* and *put* expression (3, 4, 19, 23, 46). Thus, the requirement for a high intracellular concentration of NRI~P for the activation of *hut* by nitrogen limitation should reflect the requirements for the activation of the *nac* promoter. Since this represents the switching mechanism between the top and lower levels of the cascade response to nitrogen limitation, we are interested in determining the mechanisms controlling expression from the *nac* promoter.

MATERIALS AND METHODS

DNA manipulations. All plasmid constructions were by standard methods as described before (24).

Transcription templates. The transcription templates used in this study are presented schematically in Fig. 1, 6, 9, and 11. All templates were constructed in the vector pTE103 (10) or in pAN583, which is identical to pTE103 except that it contains the multicloning site from pUC18 instead of the analogous site from pUC8 (55). The pTE103 and pAN583 vectors contain a multicloning site positioned adjacent to a strong rho-independent transcriptional terminator from bacteriophage T7. Promoters in these vectors are positioned so that transcription proceeds into the fragment derived from bacteriophage T7 and terminates at the phage terminator. The plasmids pLR100, pTH8, and pAN6 have been described previously (15, 29, 31); these contain the $glnAp_2$ promoter either with the normal complement of NRI-binding sites (pLR100 and pTH8) or lacking all NRI-binding sites (pAN6).

The details of the plasmid constructions are as follows. Plasmid pTG2 contains a 588-bp *Eco*RV fragment containing the *nac* control region from *K. aerogenes* (45) cloned into the *Sma*I site of pTE103. In pTG2, a high-affinity NRI-binding site is located at its wild-type position, centered at position −153 relative to the site of *nac* transcript initiation, and a low-affinity NRI-binding site is centered at −133.5. The sequence of the low-affinity NRI-binding site resembles that of a high-affinity site with an additional base pair inserted between the symmetrical half-sites (41) (see Results). Plasmid pJF200 is similar to pTG2 except that DNA upstream from position −253 has been removed. This was done by PCR amplification (43) of pTG2 with an upstream primer that anneals to positions −253 to −234 (5′-CCCGGGATCCGGCCTTGTCAAAGCC) and a downstream primer that anneals to positions +23 to +44 (5′-GGCATGCAAGCTTGCCTCCTGT AAGGAAGGCC). The upstream primer contains a *Bam*HI site, and the downstream primer contains a *Hin*dIII site; after digestion with these enzymes, the PCR-generated fragment was cloned into the polylinker of similarly cleaved pAN583. DNA sequencing of the PCR-generated fragment of pJF200 indicated that no mutations were introduced during the PCR amplification (not shown).

Plasmid pXH210.1 was derived from pJF200 by site-directed mutagenesis to generate an *Xho*I site between the Nac-binding site and promoter sequence without changing the spacing between these sites. The primer for this mutagenesis was 5′-GCGGCGGGCGACACTCGAGGAAATGC (mutations underlined), and the mutagenesis experiment was performed with the Altered Sites kit (Promega). (The mutagenesis protocol involved construction of an intermediate plasmid in the pSelect-1 vector.) Plasmid pXH210.2 was then constructed by insertion of the 286-bp *Sal*I-*Bam*HI fragment from pBR322 (in which the *Bam*HI site had been converted to an *Xho*I site) into the *Xho*I site of pXH210.1. This plasmid was then cleaved with *Xho*I, filled in with Klenow enzyme, and religated to give pXH210.2, in which the large insertion is 290 bp instead of 286 bp. Plasmid pJF220 was indirectly derived from plasmid pJF200 by site-specific mutagenesis designed to eliminate the low-affinity NRI-binding site without altering the spacing within the *nac* control region. The primer used in this mutagenesis experiment was 5′-GTGCGAATGTATCTCTAACAAGAGTTAC CACAATCTGCTTACCGGGC (mutations underlined). This mutagenesis was performed on the pSelect-1 construct that had already been mutagenized so as to contain the *Xho*I site, as noted above. Plasmid pJFX286 was then constructed from pJF220 by insertion of the 286-bp fragment from pBR322, as noted above for pXH210.1.

Plasmid pJF2 was constructed by inserting an *Alu*I-*Taq*I fragment from pTG2 (positions −29 to −216), to which *Eco*RI linkers had been added, into the *Eco*RI site of pAN6. In this template, the high-affinity NRI-binding site from the *nac* control region is centered at −199, and the low-affinity NRI-binding site from the *nac* control region is centered at −179.5 relative to the site of $glnAp_2$ transcript initiation. Plasmid pJF1 was constructed by cloning an *Rsa*I-*Hin*dIII fragment from pTG2 (positions −134 to +111) into the *Sma*I and *Hin*dIII sites of pAN583. Thus, this template is similar to pTG2 but lacks the high-affinity NRI-binding site and the first base pair of the low-affinity NRI-binding site.

Plasmid pJF3 was constructed in two steps as follows. The 134-bp *Sal*I frag-

ment from pAN7 (30), containing the two high-affinity NRI-binding sites from the *glnA* control region and the first 4 bp of the low-affinity site 3, was inserted into the *Sal*I site of pAN583, forming pWS2. In pWS2, the fragment containing the NRI-binding sites from the *glnA* control region is flanked by *Kpn*I sites, one of which is contributed by the polylinker of pAN583. The *Kpn*I fragment containing these binding sites from pWS2 was then cloned into the *Kpn*I site of pJF1, forming pJF3. In pJF3, the high-affinity NRI-binding sites 1 and 2 from the *glnA* control region are centered at −217 and −185 relative to the site of *nac* transcript initiation.

Plasmid pJF3BG0 was constructed by insertion of a *Bgl*I linker at the *Sma*I site in pJF3, which is located between the $glnAp_2$ and *nac* sequences. Plasmid pBX210.1 was made by site-directed mutagenesis of pJF200 to introduce a *Bgl*II site between the high-affinity NRI-binding site and the Nac site, and simultaneously the low-affinity NRI-binding site was partially destroyed. The primer used in this mutagenesis was 5′-GCACCGTTGTAGTGCGAGATCTAATGG GGCARACC (*Bgl*II site underlined). The pBX210.1 plasmid is similar to pXH 210.1 except that the distance between the high-affinity NRI-binding site and the promoter is 5 bp shorter than in pXH210.1. Plasmids pJF3BG0 and pBX210.1 were then used to construct pBX3BG0 as follows. The *Eco*RI-*Bgl*II fragment consisting of the two high-affinity NRI-binding sites from pJF3BG0 was cloned into *Eco*RI- and *Bgl*II-cleaved pBX210.1. In pBX3BG0, the two high-affinity NRI-binding sites from the *glnA* promoter are centered at −212 and −180, a *Bgl*II site is located between these NRI-binding sites and the Nac-binding site, and an *Xho*I site is located between the Nac site and the promoter.

Spacing mutations in the pXH210.1, pJF220, and pBX3BG0 vectors were made by stepwise addition of *Xho*I linkers and filling in with Klenow enzyme at the *Xho*I site, as follows: +4 (filled in), +12 (filled in and then 8-bp linker CCTCGAGG inserted), +14 (filled in and then 10-bp linker CCCTCGAGGG inserted), +16 (filled in and then 12-bp linker CCGCTCGAGCGG inserted), and +20 (the +16 construct within the linker cleaved and then filled in). Similarly, insertions in the *Bgl*II site were made by using *Bgl*II linkers of different lengths, coupled with filling in with Klenow enzyme: +4 (filled in), +12 (filled in and then 8-bp linker CAGATCTG inserted), +16 (filled in and then 12-bp linker GGAAGATCTTCC inserted), and +20 (the +16 construct within the linker cleaved and then filled in). Each of the spacing mutation plasmids bearing these subtle alterations was sequenced with a Sequenase kit (U.S. Biochemicals).

Bacteriological techniques. For mapping of the in vivo transcription start site from the *nac* promoter, wild-type *K. aerogenes* KC1043 (5, 19) was used. Cells were grown in glucose minimal medium with arginine as the sole nitrogen source (nitrogen limiting) or in LB medium (nitrogen excess) as described previously (34).

Purified proteins. Core RNA polymerase, NRI, NRII, and σ^{54} were prepared as described previously (31). Each of these proteins was greater than 90% pure, as judged by their appearance on Coomassie-stained gels. In no case is the fraction of protein that is active known. For the purification of σ^{54}, we constructed an overexpression plasmid based on the pJLA503 vector (44). This was done by introduction of an *Nde*I site at the initiation codon of the *rpoN* gene by PCR with primers that anneal to the beginning and end of the *rpoN* coding sequence (upstream primer, 5′-CCCGAATTCATATGAAGCAAGGTTTGCA ACTCAGGC; downstream primer, 5′-GGGAATTCGTCGACTCAAACGAGT TGTTTACGCTGG [based on the sequence in reference 42]). The downstream primer contains an *Eco*RI site, and the product of the amplification was cleaved with *Nde*I and *Eco*RI and ligated into similarly cleaved pJLA503.

Transcription assays. Templates were either supercoiled or linearized as indicated and used at a concentration of 10 nM. Reaction mixtures contained 50 mM Tris-Cl (pH 7.5), 10 mM $MgCl_2$, 50 mM KCl, 0.1 mM EDTA, and 1 mM dithiothreitol (DTT). Either the initiated-complex assay described previously (15, 29–31; see also reference 35) or the open-complex assay described previously (29, 35) was used, as indicated. In these assays, the template was mixed with core polymerase (50 or 100 nM, as indicated), NRII (160 nM), σ^{54} (191 or 383 nM, as indicated), the indicated amount of NRI, and either the nucleotides ATP, CTP, and GTP at 0.4 mM each (initiated-complex assay) or ATP as the sole nucleotide at 0.4 mM (open-complex assay) and incubated for 20 min at 37°C. The activation of transcription results in the formation of heparin-resistant ternary complexes stalled at positions requiring the incorporation of UTP in the initiated-complex assay (29, 35); in the open-complex assay, the activation of transcription results in the formation of heparin-resistant uninitiated complexes (29, 35). In the initiated-complex assay, a solution containing heparin (200 μg/ml final concentration) and labeled UTP (0.1 mM final concentration) was then added, and the incubation was continued at 37°C for 15 min. This results in a single cycle of extension of the transcription complexes to yield full-length transcripts. The reactions were stopped by the addition of an equal volume of stop solution (20 mM Tris-Cl [pH 7.5], 200 μg of yeast tRNA per ml, 100 mM EDTA, 1 M NaCl), and transcripts were recovered and subjected to electrophoresis and autoradiography as described previously (15). In the open-complex assay, a solution containing heparin and the nucleotides CTP, ATP, and GTP (0.4 mM final concentration each) and labeled UTP (0.1 mM final concentration) was added, and the samples were processed as described above. In several experiments, the nucleotide requirements for the formation of active transcription complexes were examined (see Results). For those experiments, various nucleotides were present as indicated at a final concentration of 0.4 mM each except for UTP, which was present as indicated at 0.1 mM. The products of these reactions were then examined by addition of a solution containing heparin and the missing nucleotide(s) (see Results).

Primer extension analysis of in vitro transcripts. RNA was prepared as in the in vitro transcription assay but with all four nucleotides present throughout the incubation and without the addition of heparin. The reaction mixtures were extracted twice with phenol-chloroform, and nucleic acids were precipitated with ethanol. The precipitated nucleic acids were resuspended in 100 μl of transcription assay buffer (15) and incubated with 30 U of RNase-free DNase I (Boehringer Mannheim) for 4 min at 37°C, again phenol-chloroform extracted, and ethanol precipitated. The precipitated RNA was dissolved in 14 μl of TE buffer (24). The primer was 5′-end labeled with polynucleotide kinase as described before (2). The primer sequence was 5′-GCTGCAGGATCGACGGATCCCC ATCGACG. This primer anneals to the multicloning site of pTG2, downstream from the *nac* promoter. The annealing reaction mixture (10 μl), containing RNA in TE buffer (6.5 μl from above), primer (about 3×10^5 cpm), and KCl (150 mM), was incubated at 90°C for 4 min, and the temperature was allowed to decrease slowly over 45 min by successive transfers to a 65°C and then a 37°C waterbath. The annealing reaction mixture was then added to a 25-μl reaction mixture containing 70 mM Tris-Cl (pH 8.3), 5 mM $MgCl_2$, 90 mM KCl, 5 mM DTT, 50 μg of bovine serum albumin (BSA) per ml, 50 U of RNasin (Promega Biotech), and 5 U of avian myeloblastosis virus reverse transcriptase (Bethesda Research Laboratories). The mixture was incubated for 60 min at 42°C, and nucleic acids were ethanol precipitated, subjected to electrophoresis on a 6% sequencing gel, and visualized by autoradiography. The same primer was used to generate the sequencing ladders shown in the control lanes, with a double-stranded supercoiled DNA template, with a Sequenase kit (U.S. Biochemicals).

Primer extension analysis of in vivo transcripts. Total RNA was isolated from bacterial strain KC1043 by hot phenol extraction (2, 16). A total of 30 μg of RNA from cells grown on nitrogen-limiting (glucose-arginine) medium and 50 μg of RNA from cells grown on nitrogen excess (LB) medium were used for the primer extension reactions. The primer in this case was 5′-CCGATATCGACGATTTT CAC, which anneals to the *nac* transcribed sequence at positions +72 to +90 relative to the site of transcript initiation. Primer labeling, annealing, extension, and the detection of extended products were done as described above.

DNase I footprinting. For footprinting the *glnA* control region, plasmid pLR100 (31) (see Fig. 11), 3′-end labeled upstream from the $glnAp_2$ promoter (31), was used. To footprint the *nac* control region, plasmid pJF200, which contains *nac* sequences from positions −253 to +44 bp relative to the site of transcript initiation, cloned into the *Bam*HI and *Hin*dIII sites of pAN583 (Fig. 11) was used. The pJF200 plasmid was cleaved with *Bam*HI and end labeled at both ends with the Klenow fragment of DNA polymerase I (24). The labeled DNA was then cleaved with *Eco*RI to produce a fragment labeled at positions −255 to −258 bp relative to the site of *nac* transcript initiation. Proteins at the indicated concentrations were incubated with DNA under the same conditions used in the transcription assay but at room temperature. The reaction mixtures were then treated with 0.24 U of DNase I (RNase-free; Boehringer Mannheim) for 2 min, and the digestion by DNase I was stopped by addition of a solution containing 50 mM EDTA, 500 mM NaCl, and 100 μg of tRNA per ml. The reaction mixtures were extracted with phenol-chloroform (1:1) and ethanol precipitated. The samples were resuspended, subjected to electrophoresis on 6% acrylamide–7 M urea sequencing gels, and autoradiographed as described previously (24). Size standards were produced by restriction enzyme digestion of the end-labeled probes. In addition, the end-labeled probes were subjected to the Maxam-Gilbert G+A chemical cleavage reaction (24) to help identify the protected sequences. These G+A lanes were cropped from the photographs and are not shown.

RESULTS

Transcription from the *nac* promoter by σ^{54} RNA polymerase required NRI and NRII. We examined the requirements for transcription from the *nac* promoter in a transcription system consisting of purified *Escherichia coli* components. The transcription templates used in this experiment are presented schematically in Fig. 1A. The formation of initiated (ternary) complexes at the *nac* and $glnAp_2$ promoters on supercoiled templates was examined (Fig. 1B). As shown, transcription from both the *nac* promoter and the previously characterized $glnAp_2$ promoter occurred in the presence of the combination of σ^{54} RNA polymerase, NRI, and the protein kinase NRII. Omission of NRI or NRII eliminated transcription from either promoter. When all protein components were present in excess, the *nac* and $glnAp_2$ promoters were of approximately equal strength, as judged by visual inspection of the intensity of the signal (Fig. 1B).

Identification of the *nac* transcription start site in vivo and in vitro. We used primer extension analysis to identify the site

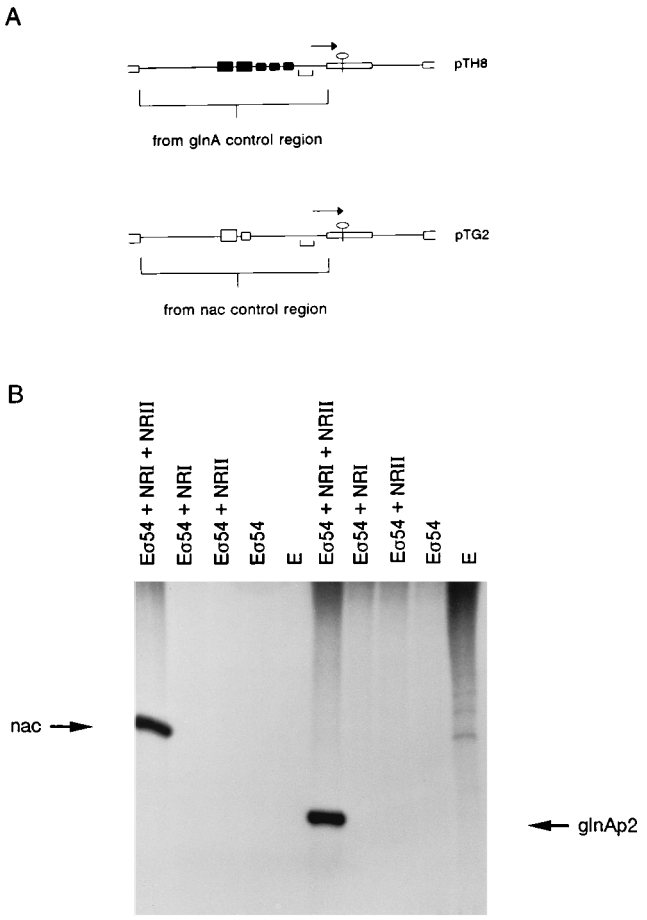

FIG. 1. Transcription from the *nac* promoter by purified bacterial components requires σ^{54} and is activated by NRI~P. (A) Schematic depiction of transcription templates containing the *nac* and $glnAp_2$ control regions. The site at which σ^{54} RNA polymerase binds is indicated by a bracket beneath the line. NRI-binding sites are indicated by boxes, with large boxes signifying high-affinity binding sites and smaller boxes signifying low-affinity binding sites. The boxes signifying NRI-binding sites from the *glnA* control region are solid, and those from *nac* are open. The stippled box with lollipop signifies the DNA fragment from bacteriophage T7, containing a strong rho-independent terminator found in the vector portion of the plasmids. The arrow signifies the transcript obtained from each plasmid. In the case of the pTH8 plasmid, this transcript measures 309 nucleotides (nt); for pTG2, the transcript measures 397 nt. The figure is not drawn to scale. (B) Initiated-complex assay of transcription from the *nac* and $glnAp_2$ promoters. Core RNA polymerase was present in all samples at 100 nM. σ^{54}, NRII, and NRI were present where indicated at 200 nM, 160 nM, and 200 nM, respectively.

of transcription initiation for the *nac* promoter both in vivo and in vitro. In intact cells, the *nac* transcript was not detected by the primer extension method with 50 μg of RNA extracted from cells grown in nitrogen excess medium, as expected, but the *nac* transcript was clearly detected in 30 μg of RNA extracted from cells grown in nitrogen-limiting medium (Fig. 2). This in vivo transcript initiated at the A residue located 25 nucleotides downstream from the highly conserved GG dinucleotide that forms part of the σ^{54}-dependent promoter sequence. Transcription in the in vitro system started primarily at this same A residue and also at the G residue 2 bp upstream (Fig. 2).

Characterization of the stability of open complexes formed at the *nac* and $glnAp_2$ promoters. In the experiment shown in Fig. 1B, we examined the formation of heparin-resistant complexes formed in the presence of the nucleotides ATP, CTP, and GTP. Previous results with the $glnAp_2$ promoter have indicated that such complexes are initiated and are rifampin resistant and contain RNA polymerase stalled at positions requiring the incorporation of UTP (29, 35). Furthermore, it has been shown that the formation of open, rifampin-sensitive, uninitiated binary complexes containing σ^{54} RNA polymerase requires the presence of ATP and that these complexes are formed in the presence of ATP as the sole nucleotide (29, 35). We examined the effect of including various combinations of nucleotides in transcription reaction mixtures containing all protein components in excess and the *nac* template. The results of these experiments were as follows. The combination AGC (Fig. 1, initiated-complex assay) gave the greatest number of *nac* transcription complexes, and the combination ACU resulted in almost the same level of transcription, as did the combination AG (not shown). Fewer transcription complexes were obtained with the combination AC, and the combination UGC resulted in very few transcription complexes, as did the combination AU. In reaction mixes containing single nucleotides (open-complex assay), only ATP resulted in the formation of appreciable numbers of transcription complexes (Fig. 3; see below); GTP resulted in barely detectable transcription, and reaction mixtures containing UTP or CTP showed no evidence of transcription complex formation (data not shown). These results suggested that the combinations ACG and ACU resulted in the formation of initiated complexes at the *nac* promoter (see below). As shown in Fig. 2 (bottom), these combinations of nucleotides would permit the formation of an RNA chain containing 3 nucleotides. Furthermore, the data suggested that our GTP solution contained some CTP and our CTP solution contained some GTP. In all subsequent experi-

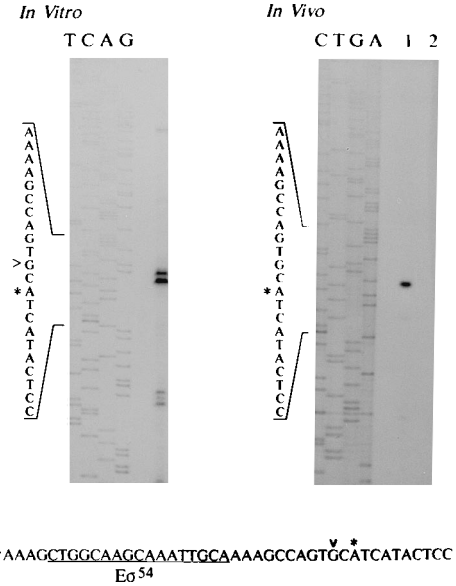

FIG. 2. Primer extension analysis of the site of transcription initiation from the *nac* promoter in vivo and in vitro. The left panel illustrates the results obtained with RNA from an in vitro transcription reaction with template pTG2. T, C, A, and G refer to the sequencing reactions with the same primer. The right panel illustrates the result obtained with RNA prepared from *K. aerogenes* grown under nitrogen-limiting (lane 1, 30 μg of RNA) and nitrogen-rich (lane 2, 50 μg of RNA) conditions. C, T, G, and A refer to sequencing reactions with the same primer. The A residue marked with an asterisk is the major site of *nac* transcription initiation. The G residue marked with a caret was only observed with RNA formed in vitro. The sequence of the *nac* promoter is shown at the bottom; the underlined portion of the sequence is closely related to the consensus sequence for σ^{54}-dependent promoters.

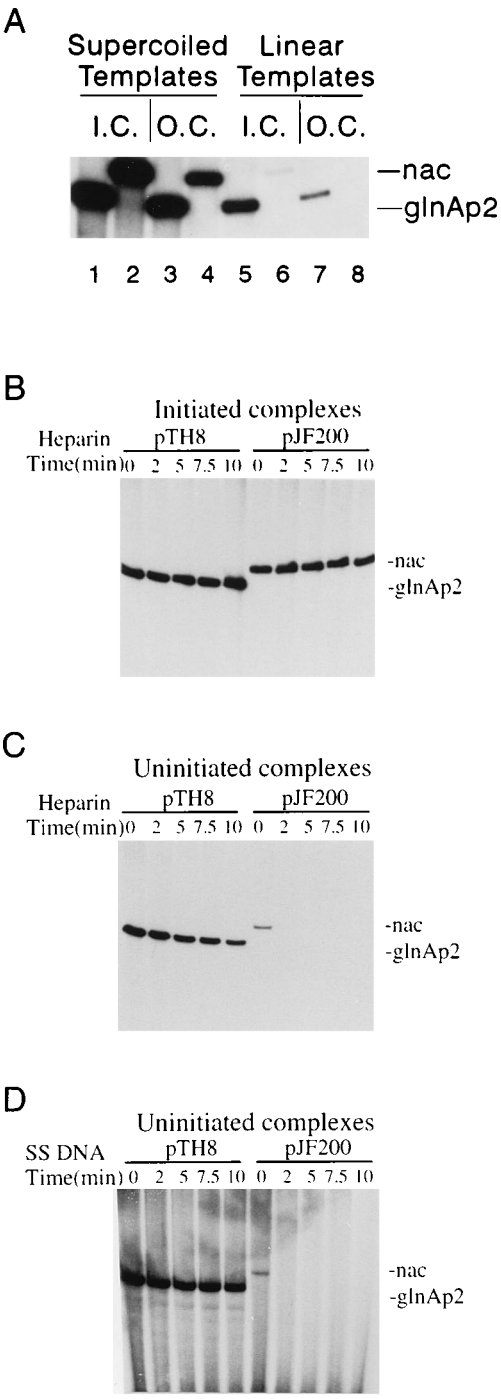

FIG. 3. Comparison of the stability of initiated and uninitiated complexes on supercoiled templates containing the *nac* and $glnAp_2$ promoters. (A) Comparison of the formation of initiated and uninitiated transcription complexes on linear and supercoiled templates containing the *nac* and $glnAp_2$ promoters. Initiated-complex (I.C.) and open-complex (O.C.) assays were performed on supercoiled templates or on linear templates that had been cleaved with *Eco*RI. The $glnAp_2$ template, pTH8, was present in lanes 1, 3, 5, and 7. The *nac* template, pJF200, was present in lanes 2, 4, 6, and 8. All reaction mixtures contained template (10 nM), core RNA polymerase (50 nM), σ^{54} (383 nM), NRI (136 nM), and NRII (160 nM). The positions of the *nac* and $glnAp_2$ transcripts are indicated. (B and C) Stability of initiated and open complexes formed at the *nac* and $glnAp_2$ promoters to heparin challenge. Initiated complexes (B) and uninitiated complexes (C) were formed on $glnAp_2$ (pTH8) or *nac* (pJF200) templates by incubation of template (10 nM) with core RNA polymerase (50 nM), σ^{54} (383 nM), NRI (136 nM), and NRII (160 nM) for 15 min at 37°C. Heparin (0.4 mg/ml final concentration) was then added, and the incubation was continued for the indicated time prior to the addition of UTP (initiated complexes) or all four nucleotides (uninitiated complexes). (D) Stability of open complexes formed at the *nac* and $glnAp_2$ promoters to challenge with salmon sperm DNA. The experiment was analogous to that shown in panel C except that the competitor was salmon sperm (SS) DNA (0.8 mg/ml) instead of heparin.

ments, the combination ACG was used to form initiated transcription complexes at the *nac* promoter, and ATP as the sole nucleotide was used to form complexes at the *nac* promoter that are assumed to be uninitiated.

We compared the formation of heparin-resistant transcription complexes formed at the *nac* and $glnAp_2$ promoters under conditions favoring or preventing initiation with both supercoiled and linearized templates. Previous results have indicated that transcription from σ^{54}-dependent promoters is stimulated by supercoiling of the template (31, 35, 52, 53); in a chloride buffer system (as was used here), transcription from the strong $glnAp_2$ promoter is considerably reduced upon linearization of the template, and transcription from the *nifLA* promoter from *K. pneumoniae* was not observed unless the template was supercoiled (52, 53). When all protein components were present in excess, both open and initiated complexes could be readily detected on supercoiled $glnAp_2$ templates, although slightly fewer open complexes were obtained (Fig. 3A). In agreement with the earlier results, when this same template was linearized by digestion with restriction endonuclease, fewer initiated complexes were obtained under the same conditions, and the number of open complexes obtained was considerably reduced (Fig. 3A). By comparison, while the number of initiated complexes obtained with the supercoiled *nac* template was similar to that obtained with the supercoiled $glnAp_2$ template, the number of open complexes obtained with the *nac* template was considerably less, even when the template was supercoiled (Fig. 3A, cf. lanes 2 and 4). When the *nac* template was linearized, initiated complexes were barely evident and open complexes were not evident (Fig. 3A, lanes 6 and 8), similar to the previous findings with the *nifLA* promoter (52, 53).

The great difference in the number of *nac* initiated complexes and *nac* uninitiated complexes obtained in experiments like that shown in Fig. 3A led us to suspect that uninitiated complexes formed at the *nac* promoter are not stable. Previous results have indicated that open complexes formed at the *nifLA* promoter of *K. pneumoniae* are less stable than those formed at $glnAp_2$ (53). We directly measured the rate of decay of uninitiated complexes formed at the $glnAp_2$ and *nac* promoters with supercoiled templates. For these experiments, either heparin (Fig. 3B and C) or salmon sperm DNA (Fig. 3D) was used as the competitor. It was empirically determined that a concentration of heparin of 0.1 mg/ml, when added prior to the addition of any proteins, was sufficient to prevent transcription from the $glnAp_2$ promoter (not shown). We formed initiated and open complexes on supercoiled *nac* and $glnAp_2$ templates in the absence of heparin and measured the number of complexes capable of giving rise to the full-length transcript that remained at various times after the addition of heparin to 0.4 mg/ml. As shown in Fig. 3B, initiated complexes formed at either promoter were entirely resistant to heparin for 10 min. In contrast, uninitiated complexes formed at these promoters were sensitive to titration by heparin, but there was a big difference in sensitivity (Fig. 3C). In the case of the $glnAp_2$ promoter, the number of uninitiated transcription complexes remaining declined steadily during the 10-min incubation in the presence of heparin, but in the case of the *nac* promoter, uninitiated complexes were barely

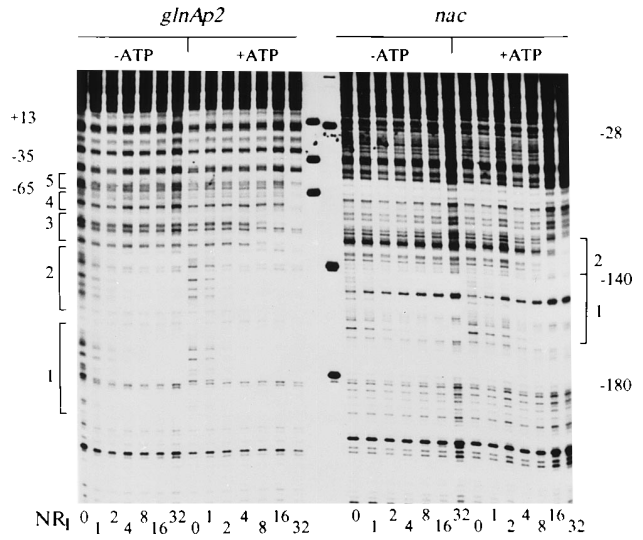

FIG. 4. DNase I footprinting of sites bound by NRI in the *glnA* and *nac* control regions. The $glnAp_2$ template was pLR100, and the *nac* template was pJF200. NRII was present in all samples at 160 nM; ATP, present as indicated, was used at 0.4 mM. In the left panel ($glnAp_2$), the concentration of NRI corresponding to 1 equals 14.4 nM. In the right panel (*nac*), the concentration of NRI corresponding to 1 equals 28.8 nM. The sites protected by NRI are labeled by numbers next to brackets. Size markers were obtained by restriction digestion of the probes. The marker positions are numbered relative to the transcription start sites.

detectable after a 2-min incubation in the presence of heparin. Thus, there was a difference in the sensitivity of open complexes formed at the *nac* and $glnAp_2$ promoters to challenge by heparin.

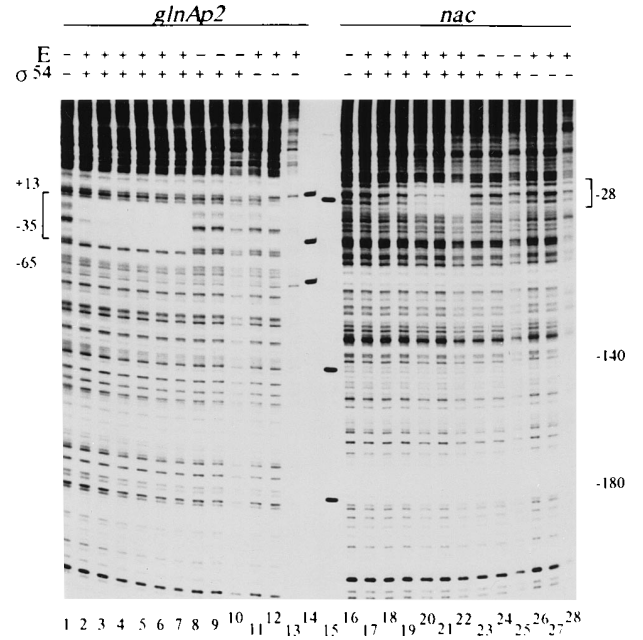

FIG. 5. DNase I footprinting of sites bound by σ^{54} RNA polymerase in the *glnA* and *nac* control regions. The samples contained σ^{54}, core RNA polymerase, or both, as indicated. Samples in lanes 2 to 7 and 17 to 22 contain both sigma 54 at 190 nM, 380 nM, 800 nM, 1.5 μM, 3.1 μM, and 6.1 μM and core RNA polymerase at 50 nM, 100 nM, 200 nM, 400 nM, 800 nM, and 1.6 μM, respectively. Samples in lanes 11 to 13 and 26 to 28 contained only core RNA polymerase, at 100 nM, 400 nM, and 1.6 μM, respectively. The samples in lanes 14 and 15 are the size markers for the $glnAp_2$ and *nac* templates.

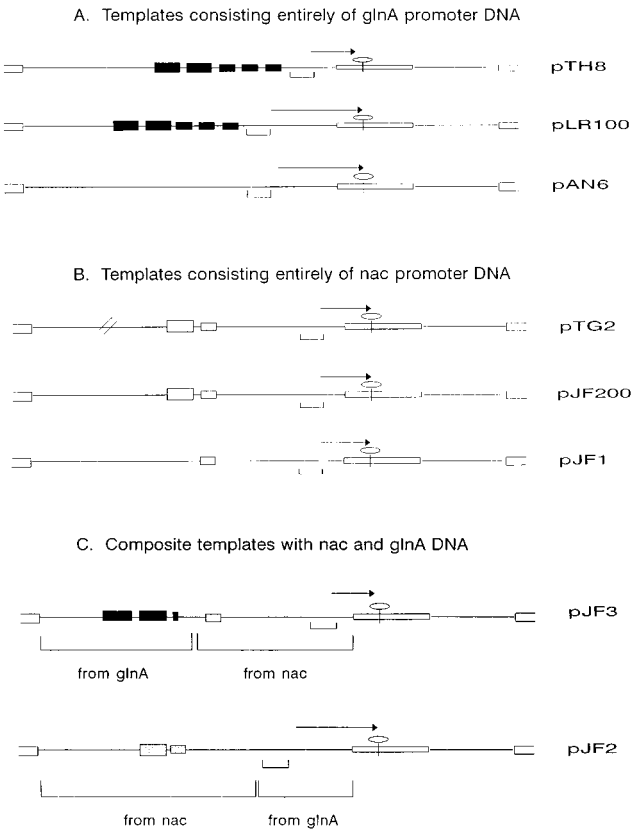

FIG. 6. Transcription templates containing various combinations of the *nac* and $glnAp_2$ promoters and NRI-binding sites. (A) Templates consisting entirely of $glnAp_2$ promoter DNA. As in Fig. 1, the bracket below the line indicates the position of the σ^{54} RNA polymerase-binding site, and the rectangles across the line represent NRI-binding sites, with high-affinity sites symbolized as larger rectangles and low-affinity sites symbolized as smaller rectangles. The vector fragment derived from bacteriophage T7 is denoted by a rectangle with a lollipop structure, indicating the phage-derived transcriptional terminator. The arrow denotes the transcript expected from each template; the pLR100 and pAN6 transcripts are 450 nt, and that from pTH8 is 309 nt. (B) Templates consisting entirely of *nac* promoter DNA. Symbols are the same as in panel A except that the NRI-binding sites are stippled instead of solid. The pJF200 transcript is 334 nt, and the transcript from pTG2 and pJF 1 is 397 nt. (C) Composite templates with *nac* and *glnA* promoter DNA. Symbols are as in panels A and B. The transcript from pJF2 is 450 nt, and that from pJF3 is 397 nt. For details of the plasmid constructions, see Materials and Methods. The figure is not drawn to scale.

To determine whether heparin was playing an active role in the dissociation of these transcription complexes, an analogous series of competition experiments with salmon sperm DNA as the competitor were conducted. It was empirically determined that 0.4 mg of salmon sperm DNA per ml, when added prior to the addition of proteins, essentially prevented transcription from $glnAp_2$ (data not shown). We conducted experiments analogous to those shown in Fig. 3B and C with salmon sperm DNA at 0.8 mg/ml in place of heparin. We found that initiated complexes at both promoters were stable to challenge by salmon sperm DNA (not shown). In contrast, open complexes formed at both promoters were sensitive to challenge by salmon sperm DNA, and again the *nac* promoter was more sensitive to challenge than was $glnAp_2$ (Fig. 3D). These results suggest that uninitiated competitor-resistant complexes, presumably open complexes, formed at both the $glnAp_2$ and *nac* promoters have a measurable rate of decay to the competitor-sensitive (presumably closed) complex and that with the *nac*

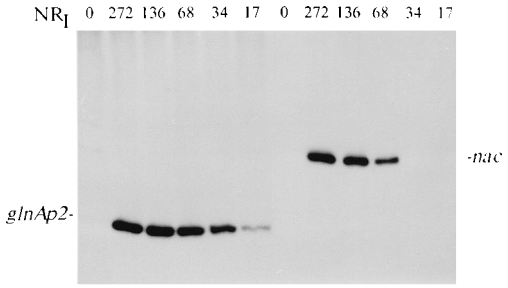

FIG. 7. Activation of the *nac* promoter requires a higher concentration of NRI~P than does activation of $glnAp_2$. Initiated-complex assay of the activation of transcription from $glnAp_2$ (pTH8 template) and *nac* (pTG2 template) promoters. Reaction mixes contained template at 10 nM, core RNA polymerase, σ^{54}, NRII at 100, 383, and 160 nM, respectively, and the indicated concentration of NRI (nanomolar).

promoter, this rate is much greater than with the $glnAp_2$ promoter and similar to the rate previously determined for the *nifLA* promoter (53).

Identification of sites protected by NRI and σ^{54} RNA polymerase in the *nac* promoter region. We examined the pattern of protection obtained in DNase I footprinting experiments when the *nac* promoter and the $glnAp_2$ promoter are incubated with various concentrations of NRI and σ^{54} RNA polymerase. In contrast to the $glnAp_2$ promoter, which contains two adjacent high-affinity NRI~P binding sites (14), the *nac* promoter region contains a single high-affinity NRI-binding site, centered at position −153 relative to the site of transcript initiation (Fig. 4). At high NRI concentration, a second site in the *nac* control region was protected by NRI, at positions −140 to −126. Examination of the nucleotide sequence of this region indicates a sequence with similarity to an NRI-binding site and with an extra base pair located between the symmetrical half-sites, centered at position −133.5. This low-affinity site was only protected by NRI when NRII and ATP were included in the reaction mixture, indicating that at these concentrations, NRI must be phosphorylated to occupy the low-affinity site. In additional experiments, we mutagenized the low-affinity site at multiple positions so as to remove any similarity to an NRI-binding site (Materials and Methods). Footprinting of this mutant promoter indicated that DNA from positions −140 to −126 was no longer protected, even when NRI~P was present at very high concentrations (data not shown).

The closed complex formed between σ^{54} RNA polymerase and the $glnAp_2$ promoter is of sufficient stability to be detected in DNase I footprinting experiments (31). We compared the ability of σ^{54} RNA polymerase to protect the $glnAp_2$ and *nac* promoters in the absence of NRI (Fig. 5). The σ^{54} RNA polymerase provided essentially complete protection of the $glnAp_2$ promoter sequence. The *nac* promoter was also protected by σ^{54} RNA polymerase in the absence of NRI, but this protection was less complete and required a higher concentration of polymerase.

Functional analysis of the NRI-binding sites at the *nac* and $glnAp_2$ promoters. We examined the dependence of initiated-complex formation at the *nac* and $glnAp_2$ promoters on the concentration of NRI under conditions in which NRII and ATP were present. The supercoiled transcription templates used in these experiments are schematically presented in Fig. 6. Transcription from the wild-type *nac* promoter required a two- to fourfold higher concentration of NRI~P than did transcription from $glnAp_2$ (Fig. 7). The same result was obtained when both promoters were present in the same transcription assay reaction mixtures (Fig. 8C). Under these conditions, transcription from the *nac* promoter was again observed only at elevated NRI concentrations.

Since the *nac* and $glnAp_2$ promoters responded differently in NRI titration experiments, we examined the role of the high-affinity NRI-binding sites in determining the sensitivity of these promoters to the NRI concentration. To do this, we tested a series of templates containing the *nac* and $glnAp_2$ promoters without upstream NRI-binding sites and with sites in various combinations (templates summarized in Fig. 6). When no high-affinity NRI-binding sites were present, neither the *nac* promoter nor the $glnAp_2$ promoter was efficiently transcribed even at high concentrations of NRI (Fig. 8E and F), even if a low-affinity site was present. When the pair of NRI-binding sites from *nac* (high affinity plus low affinity) were present, the

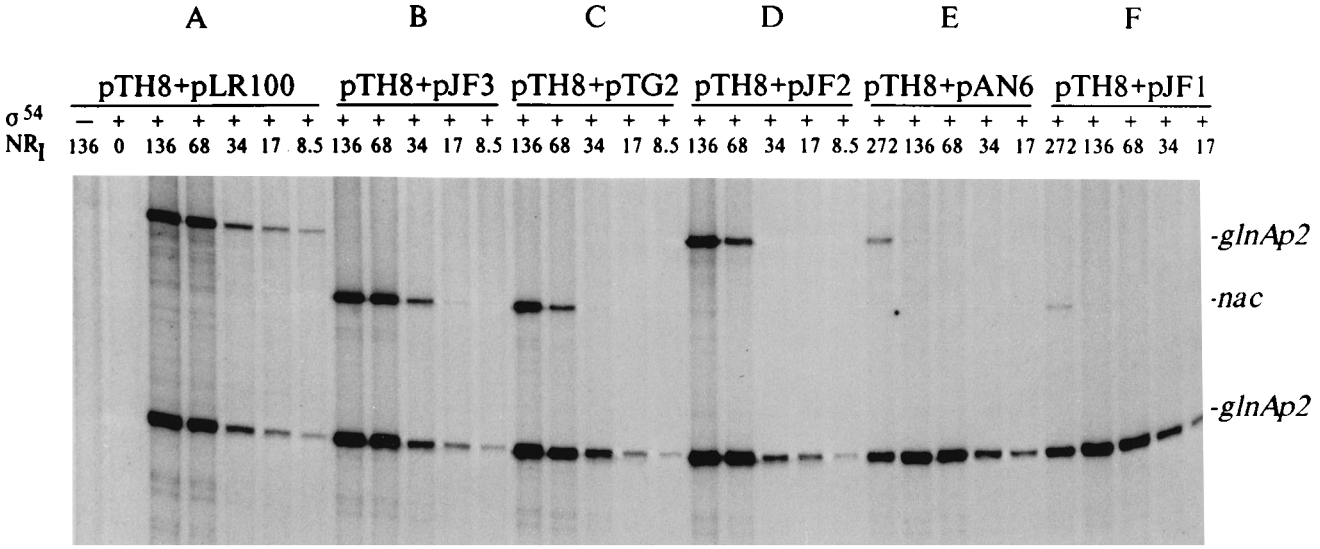

FIG. 8. Role of the NRI-binding sites in the activation of transcription from the *nac* promoter. The formation of initiated complexes in reactions with different combinations of templates was measured. Each reaction mixture contained as an internal control the $glnAp_2$ template pTH8 (10 nM) and another template at 10 nM, as indicated. Core RNA polymerase, σ^{54}, and NRII were present in all samples at 100 nM, 383 nM, and 160 nM, respectively. NRI was present as indicated (nanomolar).

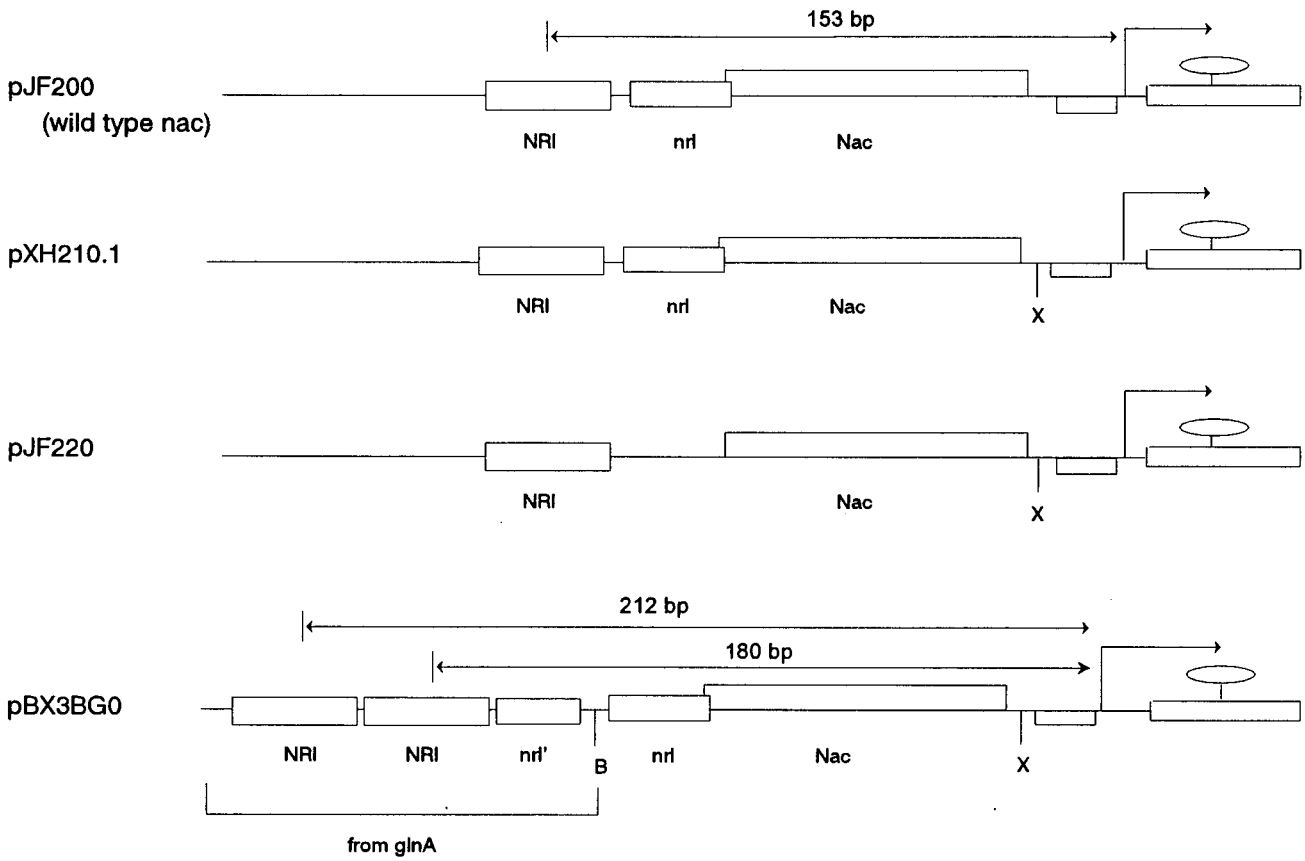

FIG. 9. Transcription templates used to study the effect of alterations in the distance between either the Nac site and promoter or the Nac site and NRI-binding sites. Symbols are as in Fig. 1 and 6 except that the Nac site is now included, denoted as a rectangle above the line (11a). NRI-binding sites are from the *nac* control region unless indicated. High-affinity NRI-binding sites are denoted NRI, and low-affinity sites are denoted nrI. X denotes the introduced *Xho*I site, and B denotes the introduced *Bgl*II site. The pJF200 transcript is 334 nt, that from pXH210.1 is 367 nt, and that from pJF220 and pBX3BG0 is 372 nt. The distances between the promoter-proximal high-affinity NRI-binding site and the site of transcription initiation are indicated. The figure is not drawn to scale.

$glnAp_2$ promoter was activated at an intermediate concentration of NRI, similar to that required to activate the wild-type *nac* promoter (cf. Fig. 8C and D). When the pair of high-affinity NRI-binding sites from $glnAp_2$ were present, the *nac* promoter was activated by a low concentration of NRI (cf. Fig. 8A, B, and C). In each of these comparisons, the construct containing the $glnAp_2$ promoter required slightly less NRI for activation than did the construct containing the *nac* promoter (for example, cf. Fig. 8A with B). Therefore, both the upstream sequences and the polymerase-binding promoter sequences contribute to the sensitivity of the *nac* promoter to NRI~P. Note that at very high concentrations of NRI~P, the activation of *glnA* was decreased in these experiments (Fig. 8E and F), as expected (47).

Role of the low-affinity NRI~P-binding site in the *nac* control region. In order to gauge the importance of the low-affinity NRI~P-binding site in the *nac* control region, we mutagenized this DNA sequence to a sequence bearing no resemblance to an NRI-binding site (Materials and Methods). We then compared the activation of initiated-complex formation on this template with that on the native *nac* template at various concentrations of NRI~P (templates pJF200 and pJF220 are schmatically illustrated in Fig. 9). As shown, elimination of the low-affinity NRI~P binding site increased by about twofold the concentration of NRI~P required to bring about the activation of transcription (Fig. 10A, pJF220 versus pJF200, 0 insertion).

Effect of subtle alterations in the position of NRI-binding sites upstream from the *nac* promoter. Many prior results have led to the model that NRI~P, bound to upstream binding sites, must contact the closed complex to activate transcription. Such an interaction would require the looping-out of the intervening DNA. Consequently, we were interested in whether the activation of transcription from *nac* promoter constructs containing various combinations of upstream NRI-binding sites had a face-of-the-helix dependency. No such face-of-the-helix dependency was previously observed for transcriptional activation from the $glnAp_2$ promoter (41); however, a clear face-of-the-helix dependency has been observed for the activation of transcription from the *nifLA* promoter of *K. pneumoniae* by NRI~P (26). This promoter, like *nac*, lacks the presence of two high-affinity NRI-binding sites that are found at $glnAp_2$ and has instead two low-affinity sites. The templates used in this analysis are schematically presented in Fig. 9.

As shown in Fig. 10C, when the upstream component of the native *nac* control region was replaced with one containing the two high-affinity NRI-binding sites from the *glnA* control region, the activation of initiated-complex formation by NRI~P required lower concentrations of NRI~P than did activation of the wild-type *nac* control template, and this activation was not much affected by subtle changes in the positions of the pair of upstream NRI-binding sites (Fig. 10C, pBX3BG0 versus pJF200). In contrast, the activation of initiated-complex formation by NRI~P with either the single high-affinity site from

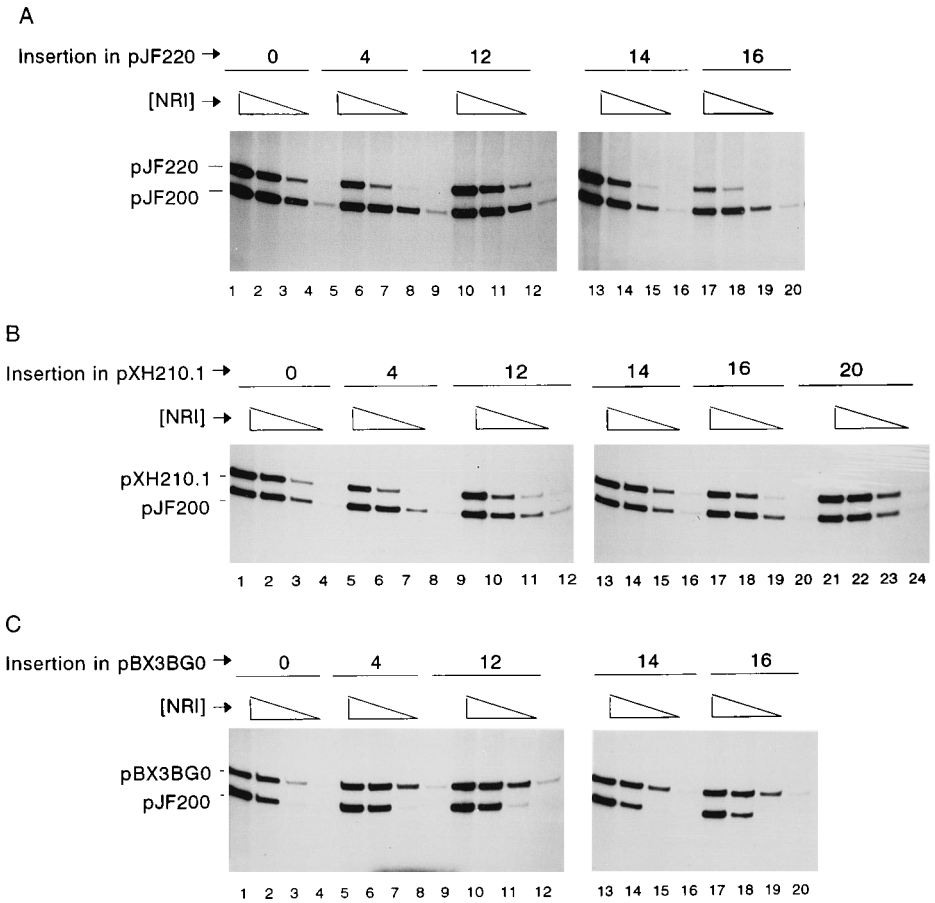

FIG. 10. Effect of subtle alterations in the position of the enhancer on activation from the *nac* promoter. The formation of initiated complexes was measured in reaction mixtures containing two templates: pJF200 as the internal control, and either pJF220, pXH210.1, or pBX3BG0 or their derivatives to be tested. The number of base pairs inserted at the *Xho*I site of the test plasmid, located between the polymerase-binding site and the Nac site, is shown. Core RNA polymerase, σ^{54}, and NRII were present at 50 nM, 383 nM, and 160 nM, respectively. The concentration of NRI within each set of data was (from left to right) as follows: 68 nM, 34 nM, 17 nM, and 8.5 nM.

the *nac* promoter (pJF220) or the normal pair of sites (one high affinity, one low affinity) from the *nac* control region (pXH210.1) was clearly affected by subtle changes in the positions of the sites (Fig. 10).

Effect of a large DNA insertion between the NRI-binding site(s) and the *nac* promoter. A set of templates similar to those used in the experiment shown in Fig. 10 but containing a large DNA fragment (either 286 or 290 bp) instead of small insertions were constructed (Fig. 11A). The activation of initiated-complex formation on these templates was then measured, comparing each template with its parent lacking the large insertion (Fig. 11B). The presence of the large DNA insertion in each case had only a minor effect on the concentration of NRI~P required to activate transcription, as shown for the constructs containing the *nac* NRI-binding sites or a single NRI-binding site in Fig. 11. Thus, while small insertions had a big effect on the activation of *nac* and a *nac* construct containing a single high-affinity site, large insertions in the same promoters had only a minor effect on activation.

DISCUSSION

The *nac* promoter is highly evolved to serve a very specific role in nitrogen regulation; it must be activated only during conditions of extreme nitrogen starvation, must be able to provide the cell with Nac protein quickly upon such starvation, and must shut off when the appropriate concentration of Nac has been produced. We used purified components to study the activation of the *nac* promoter. As for other σ^{54}-dependent promoters, this activation probably requires a contact between the oligomeric activator bound upstream and the polymerase bound at the promoter in a closed complex (reviewed in reference 17). Such contact could occur by the formation of a DNA loop, bringing these factors into proximity. If so, then any factor affecting the probability of a successful collision between polymerase and the oligomeric activator should affect the frequency of open-complex formation. Apparently, supercoiling of the template greatly facilitates the formation of the required DNA loop (31, 35, 52, 53; this work). In the case of the *nac* promoter, the probability of such successful collisions is low because of a low affinity of polymerase for the promoter, as revealed in our footprinting experiments. Also, even when the *nac* promoter was fused to upstream regions containing a pair of high-affinity NRI-binding sites, it was activated less efficiently than was the *E. coli* $glnAp_2$ promoter. Furthermore, the probability of a successful collision between the oligomeric activator and the closed complex is rendered yet more unlikely by the absence in the *nac* control region of a pair of high-affinity NRI-binding sites to facilitate the oligomerization of the activator. Instead, the *nac* control region contains a single

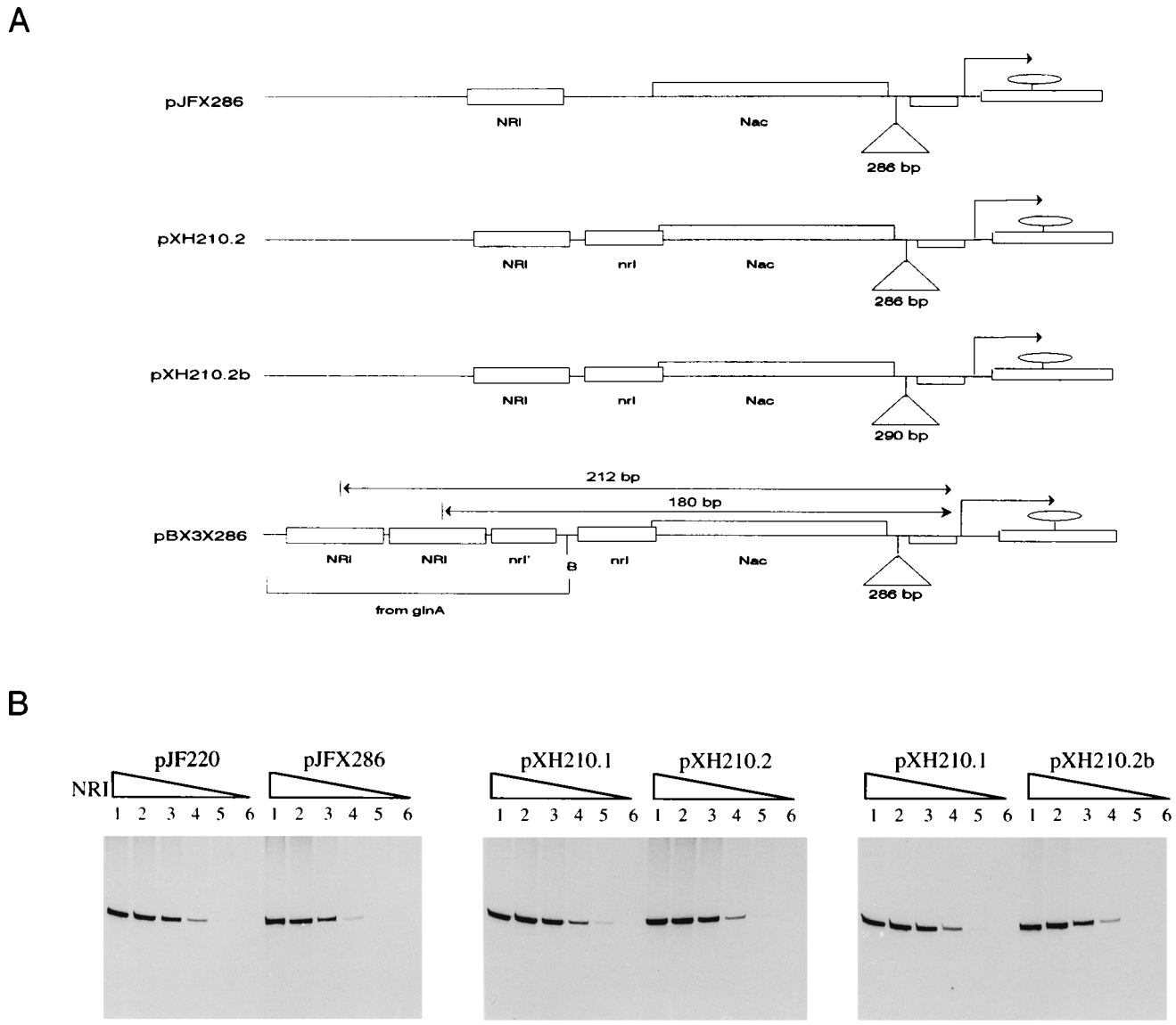

FIG. 11. Transcription from the *nac* promoter is not sensitive to the presence of a large DNA insertion between the promoter and enhancer. (A) Transcription templates containing large insertions between the enhancer and the *nac* promoter. Symbols are as in Fig. 1, 6, and 9; high-affinity NRI-binding sites are indicated as NRI, and low-affinity NRI-binding sites are indicated as nrI. The 286-bp *Bam*HI-*Sal*I fragment from pBR322 was inserted at the *Xho*I site as indicated. Templates pJFX286 and pBX3X286 produce transcripts of 367 nt, and plasmids pXH210.1 and pXH210.2 produce transcripts of 372 nt. (B) Initiated-complex assay of templates containing or lacking a large insertion between the promoter and enhancer. In all samples, core RNA polymerase, σ^{54}, and NRII were present at 50 nM, 383 nM, and 160 nM, respectively. The concentration of NRI in lanes 1 to 6 of each data series was as follows: 136 nM, 68 nM, 34 nM, 17 nM, 8.5 nM, and 4.3 nM.

high-affinity site and an adjacent NRI-binding site with much reduced affinity. This arrangement ensures that the oligomeric activator is made available to polymerase only when NRI~P is present at high concentrations.

The combination of a weak promoter and a weak enhancer found in the *nac* control region resulted in a face-of-the-helix dependency (for activation) with regard to the location of the upstream NRI-binding sites. No such face-of-the-helix dependency was observed when the two high-affinity NRI-binding sites from *glnA* were present on transcription templates. Activation of both types of templates is likely to occur by a similar mechanism, involving DNA looping. In experiments with intact cells, it has been shown that the activation of *glnA* transcription from the wild-type pair of high-affinity NRI-binding sites occurred regardless of which face of the helix these sites were on, relative to the promoter, whereas activation of $glnAp_2$ from a very weak NRI~P-binding site (in this case, half of a high-affinity site) was strongly face-of-the-helix dependent (41). Thus, if there is a face-of-the-helix dependency for the activation of transcription from a pair of high-affinity NRI~P-binding sites, then neither our in vitro transcription assays nor the in vivo measurement of glutamine synthetase (41) is sensitive enough to permit the observation of this dependency. More likely, for templates containing a pair of high-affinity NRI~P-binding sites, some other aspect of the initiation process is limiting, such as the rate of formation of the open complex after the interaction of NRI~P and RNA polymerase. Interestingly, for those templates containing weak enhancers, subtle changes in the position of the enhancer were often far more deleterious to the activation process than was the insertion of

a large DNA fragment between the enhancer and promoter, further supporting the looping model.

Our studies demonstrate that uninitiated, competitor-resistant transcription complexes at σ^{54}-dependent promoters have a significant rate of decay to the competitor-sensitive complex and that this rate is much greater for the *nac* promoter than for the $glnAp_2$ promoter. A similar phenomenon was previously noted for the *nifLA* promoter of *K. pneumoniae* (53). Thus, expression from the *nac* promoter will be limited unless conditions favor initiation. Whether this factor plays a role in intact cells remains to be determined.

Activation of transcription from a single high-affinity site still requires the oligomeric form of the activator (36) and probably involves the cooperative binding of activator to the single high-affinity site and nearby cryptic sites (51). In theory, the level of expression of a σ^{54} promoter can be optimized by mutations affecting the quality of such adjacent cryptic sites, which need only be nearby and on the same face of the helix (40, 41). In the extreme case, it could be imagined that a single high-affinity NRI-binding site alone could serve as the enhancer, with the NRI~P dimer bound there serving as the sole point of specific association for a second NRI~P dimer that entirely lacks specific contact with DNA (36, 41). However, this arrangement is clearly not seen at the *nac* promoter, since our DNase I footprinting assays revealed specific contacts of NRI~P with a low-affinity site found adjacent to the single high-affinity site. Furthermore, our transcription assays revealed that mutation of this low-affinity site to a sequence bearing no resemblance to an NRI~P-binding site resulted in a ~2-fold increase in the concentration of NRI~P required to activate the *nac* promoter. By comparison, the *nifLA* promoter of *K. pneumoniae* contains two tandem low-affinity NRI~P-binding sites (26, 54); DNase I protection of either of these sites required NRI~P at high concentrations, and mutagenesis of one of these sites increased the concentration of NRI~P required for transcriptional activation approximately twofold (26). The arrangement of high-affinity and low-affinity activator-binding sites at *nac* is more reminiscent of the arrangement of upstream DctD-binding sites at the *dctA* promoter, except that in that case, the promoter-proximal site is the high-affinity site (18). Apparently, the normally dimeric DctD protein must, like NRI, become oligomerized upon phosphorylation.

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