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Covalent modification of the *glnG* product, NR_I, by the *glnL* product, NR_{II}, regulates the transcription of the *glnALG* operon in *Escherichia coli*

(glutamine synthetase/phosphorylation/nitrogen metabolism/positive control)

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ABSTRACT **Transcription from nitrogen-regulated promoters, such as *glnAp2*, requires the *glnG* gene product, NR_I, as well as the *rpoN*(*glnF*) gene product, σ^{60}, and is regulated by the *glnL* gene product, NR_{II}. We find that in a reaction mixture containing NR_I, NR_{II}, and ATP, NR_{II} catalyzes the transfer of the γ phosphate of ATP to NR_I. This covalent modification of NR_I occurs concurrently with the acquisition of the ability by the reaction mixture to activate transcription from *glnAp2*. In the presence of P_{II}, the product of *glnB*, NR_{II} catalyzes the removal of the phosphate from NR_I-phosphate. This reaction occurs concurrently with the loss by the reaction mixture of the ability to activate transcription from *glnAp2*. On the basis of this evidence, we propose that NR_I-phosphate activates transcription from nitrogen-regulated promoters and that the role of NR_{II} is control of the formation and breakdown of NR_I-phosphate in response to cellular signals of nitrogen availability.**

In *Escherichia coli* and other enteric bacteria, transcription of the *glnALG* operon, which contains the structural gene for glutamine synthetase (*glnA*), is activated in response to nitrogen starvation at the promoter *glnAp2* (1). This activation requires the DNA-binding protein NR_I, the product of *glnG*, as well as σ^{60}, the product of *rpoN*(*glnF*), and is regulated by NR_{II}, the product of *glnL* (2–8). Wild-type cells are able to decrease or increase very rapidly the rate of transcription initiation at *glnAp2* in response to the addition or removal of ammonia. Mutants that lack NR_{II} lack the ability for this rapid response. Nonetheless, these mutants have regulated levels of nitrogen-regulated gene products in the steady state, indicating that a slower and less efficient NR_{II}-independent mechanism for the regulation of transcription from *glnAp2* does exist.

The regulation of *glnA* expression by NR_{II} requires the products of two additional genes, *glnD* and *glnB* (9, 10). The *glnD* gene product is a uridylyltransferase (UTase) required for the conversion of P_{II}, the *glnB* gene product, to a uridylylated form, and a uridylyl-removing enzyme, which catalyzes the reverse reaction. The ability of UTase to convert P_{II} to P_{II}-UMP is stimulated by 2-ketoglutarate and, conversely, the ability of uridylyl-removing enzyme to remove the uridylyl group from P_{II}-UMP is stimulated by glutamine (11). Thus, ammonia starvation, which results in a high intracellular ratio of 2-ketoglutarate to glutamine, causes the conversion of P_{II} to P_{II}-UMP. Growth with ammonia excess, which results in a high intracellular ratio of glutamine to 2-ketoglutarate, causes the conversion of P_{II}-UMP to P_{II}.

Genetic analysis has shown that the effects of P_{II} and UTase on *gluA* expression are mediated by NR_{II}. The loss of P_{II} results in the NR_{II}-dependent activation of transcription at *glnAp2* in cells grown with an excess of nitrogen and, conversely, the loss of UTase results in the inability to activate transcription from *glnAp2* (10). The effects of all mutations in *glnB* and *glnD* are suppressed, with regard to the steady-state levels of nitrogen-regulated gene products, by mutations resulting in the loss of NR_{II} (4, 10). Mutations in *glnL* can result in an altered product, such as $NR_{II}2302$, that causes the activation of transcription at nitrogen-regulated promoters without reference to the availability of nitrogen and the presence or absence of P_{II} and UTase (4, 5).

On the basis of these observations, it has been proposed that in the presence of P_{II}, NR_{II} converts NR_I to a form incapable of activating the initiation of transcription at nitrogen-regulated promoters. In the absence of P_{II}, brought about by its UTase-catalyzed conversion to the innocuous P_{II}-UMP, NR_{II} converts inactive NR_I to the form capable of activating transcription from nitrogen-regulated promoters (10).

Recent work in this laboratory has included the study of the transcription of *glnA* by purified components of *E. coli* (8). It was shown that the initiation of transcription at *glnAp2* carried on a supercoiled template requires core RNA polymerase and σ^{60}. This transcription was greatly stimulated by the addition of NR_I and $NR_{II}2302$. Neither NR_I nor $NR_{II}2302$ caused any stimulation of transcription when added alone.

In this paper, we describe additional experiments with purified components of *E. coli*. Their results indicate that NR_{II} regulates the activity of NR_I by covalent modification.

MATERIALS AND METHODS

Purified Proteins. The product of the *rpoN* gene, σ^{60}, was purified as described by Hunt and Magasanik (8). NR_I was provided by Lawrence Reitzer (6), core RNA polymerase was provided by Tom Hunt, and P_{II} was provided by S. G. Rhee (12).

The products of the *glnL* gene, NR_{II}, and of a mutant *glnL* allele, $NR_{II}2302$, were purified by a procedure that will be described in detail elsewhere. For both proteins, we used high-copy-number plasmids containing a previously described promoter-up mutation at *glnLp* (13, 14). The purification steps included chromatography on agarose-ethane and DEAE-Sephacel for NR_{II}, and these two steps as well as gel filtration using AcA44 for $NR_{II}2302$. In each case, the purified protein was ≈90% pure as judged by $NaDodSO_4$/polyacrylamide gel electrophoresis.

Transcription Assays. The conditions and template plasmid were as described (8) with the following modifications: supercoiled template DNA was present at 5–10 nM, NR_I was added to 240 nM, core RNA polymerase (E) was added to 52 nM, and σ^{60} was added to 175 nM. NR_{II} and $NR_{II}2302$ were

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Abbreviation: UTase, uridylyltransferase.

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added as indicated. To determine the time course of the formation of the open RNA polymerase–promoter complex (18) (see Fig. 2A), a reaction mixture 6.5 times normal size was assembled lacking NR_{II}, UTP, and heparin. These components were prewarmed for 2 min at 37°C, followed by the addition of NR_{II}. At designated times, aliquots were removed and UTP and heparin were added. The experiments in which NR_I was pretreated with NR_{II} (Figs. 2 and 3) were performed as follows: reaction mixtures 3.5 times normal size containing all components except NR_I, NR_{II}, UTP, and heparin were assembled and held on ice. Each of these reaction mixtures was warmed for 5 min at 37°C prior to the addition of a mixture of NR_I and NR_{II} or $NR_{II}2302$. After this addition (time 0), aliquots were removed at the designated times and processed to determine the time course of open complex formation. The mixture of NR_I and NR_{II} contained additional components as indicated and was assembled on ice with NR_{II} added last. For the experiment in Fig. 2B, samples of this mixture were tested immediately after the addition of NR_{II} ("no pretreatment"), and after 20 min at 37°C ("pretreated"). For the experiments shown in Fig. 6 C and D, 1-μl samples of the protein-labeling reaction mixtures containing NR_I and NR_{II} or $NR_{II}2302$ (see below) were mixed with the other components, which had been warmed for 2 min, and 3 min was then allowed for the formation of the open complex. These transcription assays contained NR_I at 48 nM and NR_{II} at 0.8 nM or $NR_{II}2302$ at 0.2 nM. This NR_I concentration is not limiting (unpublished observation).

Protein-Labeling Experiments. The time course of ^{32}P incorporation into trichloroacetic acid-precipitable material (see Figs. 4–6) was examined in reactions containing 50 mM Tris·HCl (pH 7.8), 50 mM KCl, 1 mM dithiothreitol, 0.5 mM EDTA, 10 mM $MgCl_2$, NR_I (at 2.4 μM), 0.4 mM ATP (9000 cpm/pmol, either α- or γ-labeled), and NR_{II} or $NR_{II}2302$ as indicated. All of the components except ATP were mixed on ice and prewarmed for 2 min at 37°C. The reactions were started by the addition of ATP.

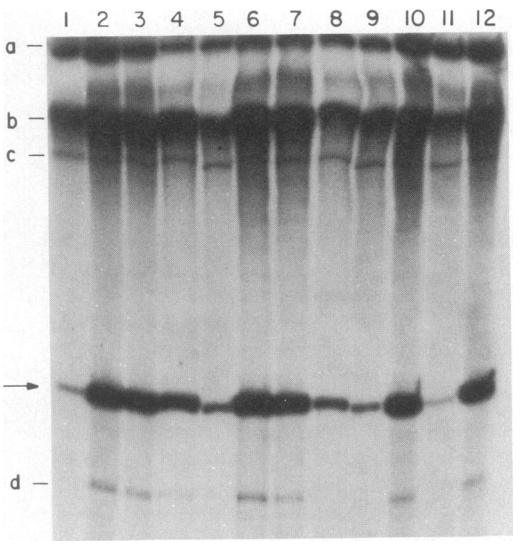

FIG. 1. Titration of NR_{II} and $NR_{II}2302$ activity. Each transcription reaction contained E, σ^{60}, NR_I, and the following concentrations of NR_{II} or $NR_{II}2302$: lane 1, no NR_{II}; lanes 2–5, NR_{II} at 10, 5, 2, and 1 nM; lanes 6–9, $NR_{II}2302$ at 5, 2.5, 1, and 0.5 nM; lane 10, $NR_{II}2302$ at 10 nM; lane 11, NR_{II} at 20 nM but heated to 90°C for 5 min prior to use; lane 12, NR_{II} at 20 nM. The position of the 309-nucleotide transcript initiated at *glnAp2* is indicated by an arrow. a, top of the gel; b, unknown transcripts, at least some of which seem to result from transcription initiated at *glnAp2* proceeding beyond the terminator; c, unknown transcript not initiated at *glnAp2*; d, unknown transcript, probably a degradation product of transcript initiated at *glnAp2*.

Samples were withdrawn at the indicated times and spotted onto Schleicher & Schuell no. 34 glass fiber filters, which were immediately immersed into ice-cold 10% trichloroacetic acid containing 1% sodium pyrophosphate (wt/vol) (PP_i). After all samples had been collected, the filters were washed for 30 min on ice in 10% trichloroacetic acid 1% PP_i, for 30 min on ice in 5% trichloroacetic acid/1% PP_i, and twice for 30 min in 5% trichloroacetic acid/1% PP_i at room temperature. The filters were then rinsed in ethanol, dried, and counted by liquid scintillation using Fisher Scintiverse I scintillation fluor.

For the analysis of labeled protein by polyacrylamide gel electrophoresis (see Fig. 5), 8-μl samples were added directly to 500 μl of 10% trichloroacetic acid/1% PP_i on ice. The samples were held on ice for 20 min and spun for 30 min in an Eppendorf centrifuge at 4°C. The recovered precipitate was dissolved in protein gel denaturing sample buffer, neutralized with NaOH, and analyzed on a 10% $NaDodSO_4$/polyacrylamide gel with a 3% stacking gel.

RESULTS

Activation of Transcription at *glnAp2*. We examined the ability of purified NR_{II} and $NR_{II}2302$ to stimulate transcription from *glnAp2* in the presence of NR_I, σ^{60}, and core RNA polymerase. In these experiments, the reaction mixtures (complete except for UTP) containing different amounts of NR_{II} or $NR_{II}2302$ were incubated for 7 min, after which transcription was initiated by the addition of UTP (Fig. 1). The amount of RNA resulting from the initiation of transcription at *glnAp2* became constant with increasing concentrations of NR_{II} or $NR_{II}2302$ and was approximately half-maximal when NR_{II} and $NR_{II}2302$ were 1.5 and 1.0 nM, respectively. Heating either NR_{II} or $NR_{II}2302$ to 70°C for 5 min completely destroyed the ability to stimulate transcription (data not shown).

We observed that suboptimal amounts of NR_{II} or $NR_{II}2302$ could bring about maximal stimulation of transcription when

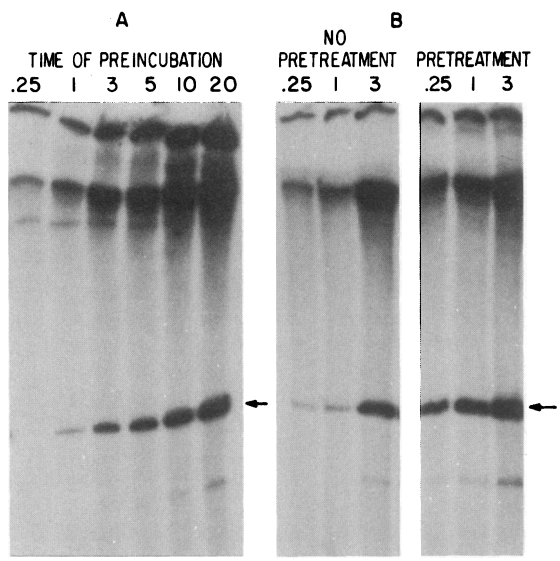

FIG. 2. (A) Time course of open complex formation for a transcription reaction containing NR_{II} at 2 nM. The number above each lane indicates the time in minutes of incubation with NR_{II} before addition of UTP and heparin. (B) The effect of pretreating NR_I with NR_{II} in the presence of ATP, CTP, and GTP for 0 min at 4°C (no pretreatment) or for 20 min at 37°C (pretreated) on the time course of open complex formation. All reactions contained NR_{II} at 2 nM and the same final concentration of nucleotides. The number above each lane refers to the time in minutes of incubation with NR_I and NR_{II} before addition of UTP and heparin. Arrow indicates the transcript initiated at *glnAp2*.

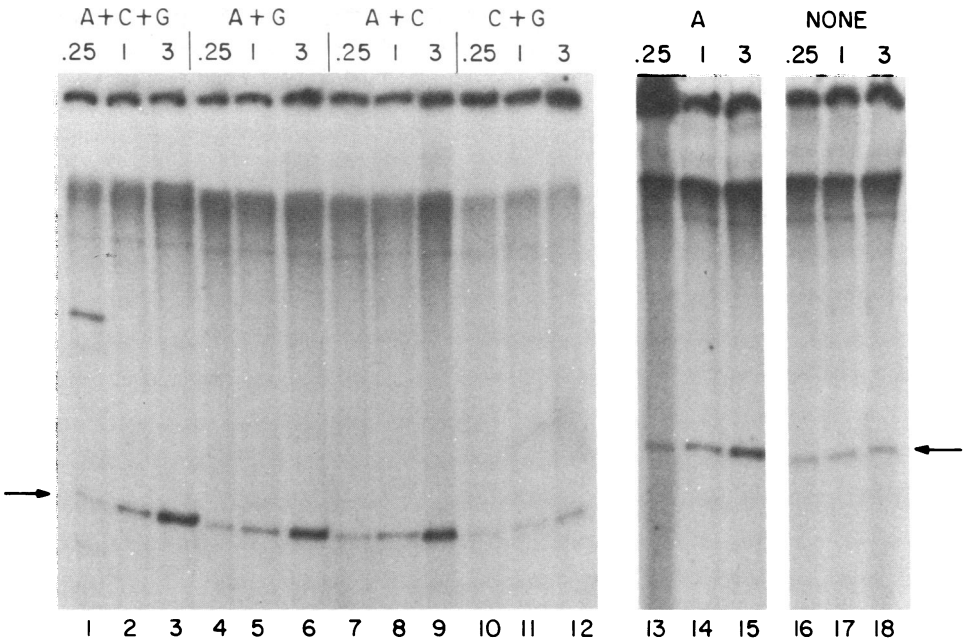

FIG. 3. Effect of pretreating NR_I with $NR_{II}2302$ in the presence of various nucleotides on the time course of open complex formation. The time course for six transcription assays containing identical amounts of $E\sigma^{60}$, NR_I, and $NR_{II}2302$ is shown. The number above each lane indicates the time of incubation with NR_I and $NR_{II}2302$ before addition of UTP and heparin. In each case, NR_I had been pretreated with $NR_{II}2302$ for 20 min at 37°C in the presence of the following nucleotides: lanes 1–3, ATP, CTP and GTP; lanes 4–6, ATP and GTP; lanes 7–9, ATP and CTP; lanes 10–12, CTP and GTP; lanes 13–15, ATP; lanes 16–18, no nucleotides. All reactions contained $NR_{II}2302$ at 1 nM. Lanes 1–12 and 13–18 are from two different gels.

the time of incubation was increased sufficiently (shown for NR_{II} in Fig. 2*A*). We examined the possibility that NR_{II} was catalyzing the conversion of NR_I to an active form during the incubation and found that pretreatment of NR_I with NR_{II} or $NR_{II}2302$ for 20 min at 37°C in the presence of ATP, CTP, and GTP significantly shortened the period of time required for maximal open complex formation (shown for NR_{II} in Fig. 2*B*). In the absence of nucleotides, the pretreatment was ineffective (not shown). In Fig. 3, we present the result obtained when the time course of open complex formation was examined in transcription assays containing NR_I pre-

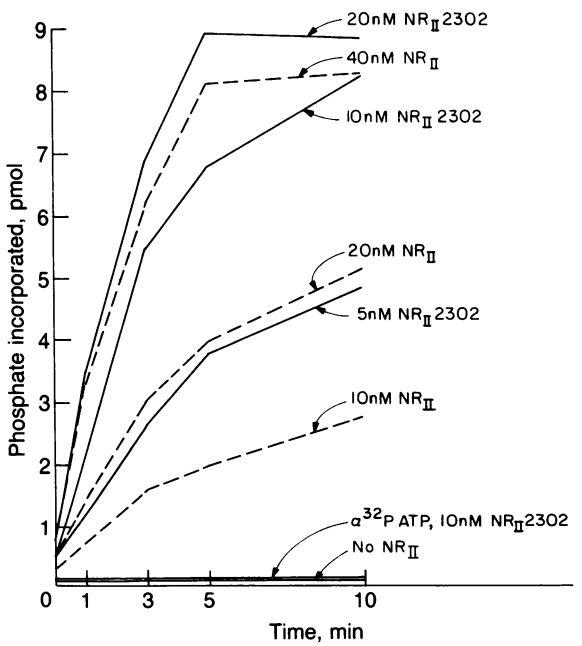

FIG. 4. NR_{II} and $NR_{II}2302$ catalyze the incorporation of the γ phosphate of ATP into protein. The time course of the incorporation of labeled phosphate into trichloroacetic acid-precipitable material is shown for reactions containing various amounts of NR_{II} or $NR_{II}2302$, NR_I at 2.4 μM and ATP at 0.4 mM. The ATP was labeled in the γ position except where noted. Each sample assayed contained 21.6 pmol of NR_I.

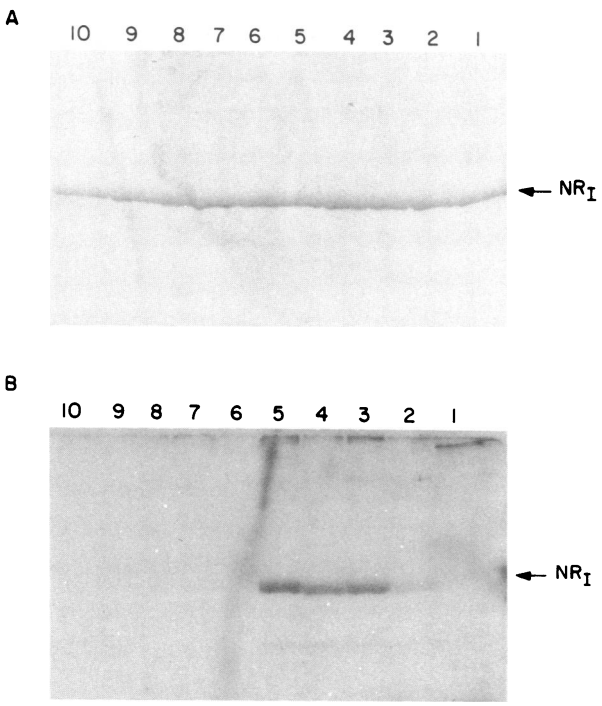

FIG. 5. Covalent modification of NR_I by $NR_{II}2302$. NR_I was incubated with [γ-^{32}P]ATP for various times in the presence or absence of $NR_{II}2302$ (10 nM). (*A*) Samples were precipitated with trichloroacetic acid, run on a $NaDodSO_4$/polyacrylamide gel, and stained with Coomassie brilliant blue. (*B*) Autoradiograph of the gel shown in *A*. Lanes 1–5, $NR_{II}2302$ present and samples removed at 0.25, 1, 3, 5, and 10 min; lanes 6–10, $NR_{II}2302$ absent and samples removed at 0.25, 1, 3, 5, and 10 min. The position of NR_I is indicated.

treated with $NR_{II}2302$ in the presence of various nucleotides. In this figure, pretreatment in the absence of nucleotides (Fig. 3) serves as a control for the effectiveness of the pretreatment. As shown, only ATP was required. Taken together, these data suggest that the rate-limiting step in the activation of transcription initiation at *glnAp2* when NR_{II} is limiting is a direct interaction between NR_I and NR_{II} requiring ATP.

***In Vitro* Modification of NR_I by NR_{II} and $NR_{II}2302$.** We examined the possibility that the ATP requirement in the "pretreatment reaction" reflected modification of NR_I by NR_{II} through nucleotidylation or phosphorylation. For this purpose, a relatively large amount of NR_I was incubated with NR_{II} or $NR_{II}2302$ in the presence of either [α-^{32}P]ATP or [γ-^{32}P]ATP. At various times after the addition of ATP, samples were removed and the incorporation of ^{32}P into trichloroacetic acid-precipitable material was measured. The results of these experiments indicate that the γ phosphate of ATP is incorporated into protein, whereas the α phosphate is not (Fig. 4). Also shown in Fig. 4 are the results obtained when various amounts of NR_{II} and $NR_{II}2302$ were incubated with NR_I and [γ-^{32}P]ATP. Using the initial reaction rates, the specific activities were 5.88 and 16.8 pmol of phosphate incorporated per min per pmol of NR_{II} and $NR_{II}2302$, respectively. Thus, in this assay the $NR_{II}2302$ preparation is $\approx$3 times as active as the NR_{II} preparation. No incorporation of the γ phosphate of ATP into protein was observed when NR_I was incubated with ATP in the absence of NR_{II} or $NR_{II}2302$. The maximal amount of phosphate incorporated corresponded to 0.45 molecule per NR_I dimer. We observed that 72% of the phosphate incorporated into protein was released by heating the sample to 98°C for 7 min in 10% trichloroacetic acid.

We examined the products of protein labeling reactions on a $NaDodSO_4$/polyacrylamide gel and by autoradiography. As shown in Fig. 5, NR_I became labeled when incubated with $NR_{II}2302$ and ATP, but it was not labeled when incubated with ATP in the absence of $NR_{II}2302$. This result indicates that the trichloroacetic acid-precipitable phosphate is covalently attached to NR_I.

Correlation Between the Covalent Modification of NR_I and

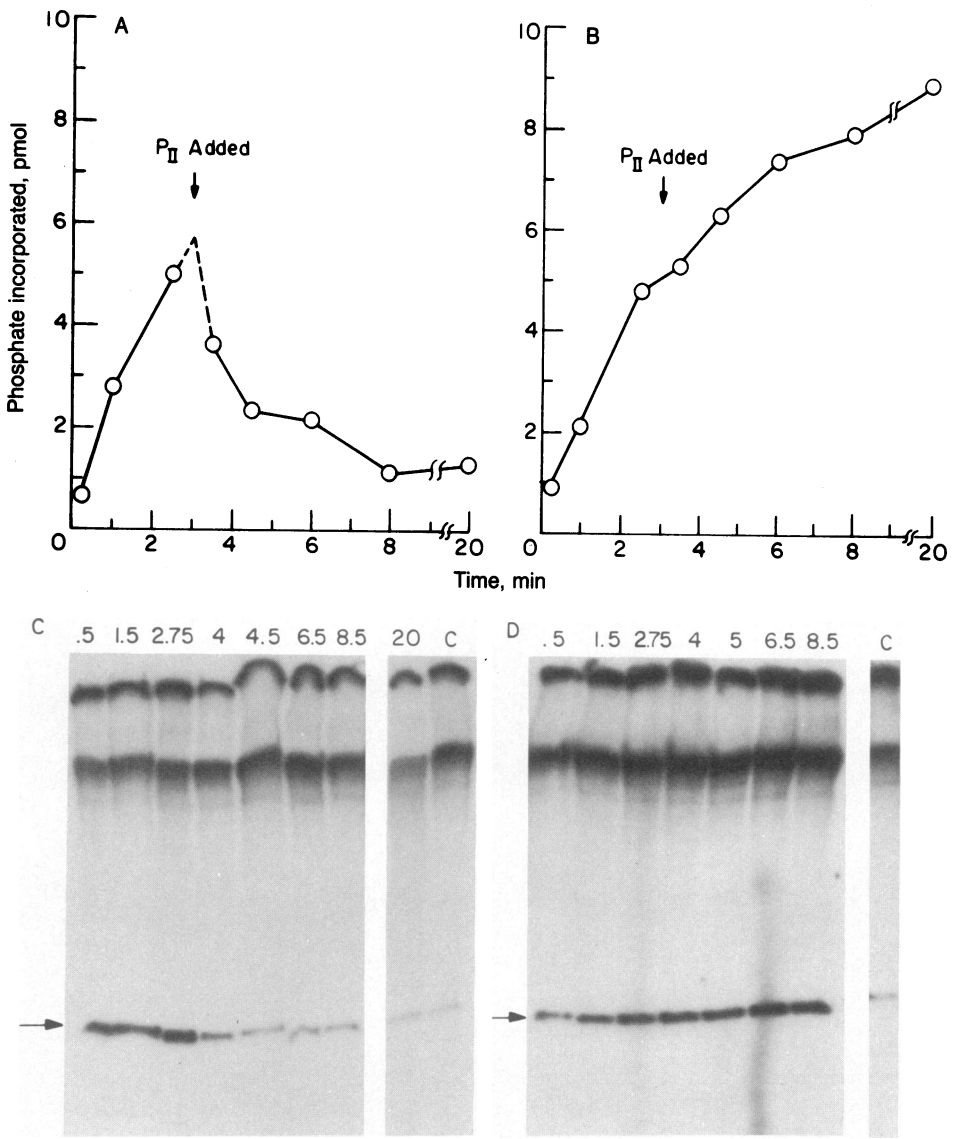

FIG. 6. Correlation between the covalent modification of NR_I and the activation of transcription at *glnAp2*. (*A*) Time course of incorporation of phosphate into protein, as in Fig. 4, using 40 nM NR_{II}. At 3 min, P_{II} was added to 0.33 μM, indicated by an arrow. Each sample analyzed contained 19.2 pmol of NR_I. (*B*) As in *A*, but $NR_{II}2302$ at 10 nM in place of NR_{II}. (*C*) Transcription assay using samples removed from the labeling reaction shown in *A* at the time indicated above each lane. The position of the transcript initiated at *glnAp2* is indicated by an arrow. Lane C is a control transcription reaction run in the same experiment that did not receive a sample containing NR_I and NR_{II}. (*D*) Transcription assay using samples removed from the labeling reaction shown in *B* at the time indicated above each lane. Lane C is a control transcription reaction from the same experiment that did not receive a sample containing NR_I and $NR_{II}2302$.

the Activation of Transcription at *glnAp2*. We tested the effect of P_{II} on the modification of NR_I by NR_{II} and $NR_{II}2302$. In the experiments shown in Fig. 6, the modification of NR_I by NR_{II} and $NR_{II}2302$ was allowed to proceed to a point where a significant amount of NR_I had been converted to NR_I-phosphate and P_{II} was then added. When NR_{II} was used in such an experiment, the addition of P_{II} caused the rapid removal of the phosphate from NR_I (Fig. 6*A*). When $NR_{II}2302$ was used in place of NR_{II}, the addition of P_{II} did not prevent the continuing incorporation of phosphate (compare Fig. 4 and Fig. 6*B*). These results suggest that in the presence of P_{II}, NR_{II}, but not $NR_{II}2302$, can dephosphorylate NR_I-phosphate.

We exploited the different response of NR_{II} and $NR_{II}2302$ to P_{II} to demonstrate indirectly that NR_I-phosphate is required for the activation of transcription from *glnAp2*. Samples of the protein-labeling reactions shown in Fig. 6 *A* and *B* were removed at various times and tested for their ability to activate transcription from *glnAp2*. Removal of the phosphate from NR_I-phosphate by NR_{II} in the presence of P_{II} simultaneously resulted in the loss of the ability to activate transcription at *glnAp2* (Fig. 6*C*). When $NR_{II}2302$ was used in place of NR_{II}, the addition of P_{II} did not diminish the activation of transcription from *glnAp2* (Fig. 6*D*).

DISCUSSION

We have shown that the activation of NR_I—that is, its conversion to the form capable of stimulating the initiation of transcription at *glnAp2*—requires NR_{II} and ATP. This conversion results from the NR_{II}-catalyzed phosphorylation of NR_I. The position of the phosphate on NR_I and the nature of the phosphate-protein bond remain to be determined.

The conclusion that NR_I-phosphate is the active form is strongly supported by the comparison of the activities of NR_{II}, the product of the wild-type *glnL* gene, and of $NR_{II}2302$, the product of the mutant gene *glnL2302*. It had previously been shown that in intact cells containing $glnL^+$, but not in those containing *glnL2302*, P_{II} could prevent the activation of transcription from *glnAp2* (4, 10). We have now shown that addition of P_{II} to a reaction mixture containing NR_{II}, NR_I-phosphate, and ATP resulted simultaneously in the release of protein-bound phosphate and in the loss of the capacity to stimulate the initiation of transcription at *glnAp2*. By contrast, when $NR_{II}2302$ was used in place of NR_{II}, the addition of P_{II} caused neither the release of protein-bound phosphate nor the loss of that capacity.

These results suggest that NR_{II} is an NR_I kinase that can be converted by P_{II} to an NR_I-phosphate phosphatase. These two activities account for the role of NR_{II} in the cyclic cascade system responsible for the regulation of the synthesis of glutamine synthetase in response to the availability of nitrogen. When the ammonia concentration is low, UTase converts P_{II} to P_{II}-UMP, NR_{II} converts NR_I to NR_I-phosphate, and transcription of *glnA* is initiated. An increase in the concentration of ammonia causes the uridylyl-removing enzyme to remove the uridylyl group from P_{II}, which in combination with NR_{II} removes the phosphate from NR_I and halts the initiation of *glnA* transcription. It has long been known that P_{II} stimulates the adenylylation and consequent inactivation of glutamine synthetase by adenylyltransferase (ATase) and that P_{II}-UMP stimulates the deadenylylation and consequent activation of glutamine synthetase by ATase (reviewed in ref. 15). Thus, P_{II} and UTase are members of two cyclic cascade systems: one responsible for the regulation of glutamine synthetase activity, and the other one responsible for the regulation of glutamine synthetase synthesis. In both cases, P_{II} causes the response appropriate for nitrogen excess: glutamine synthetase is inactivated and its synthesis is halted. However, the biochemical reactions stimulated by P_{II} are quite different: ATase is stimulated to add adenylyl groups to glutamine synthetase, whereas NR_{II} is stimulated to remove the phosphate group from NR_I-phosphate.

NR_I resembles other regulatory proteins of *E. coli*, such as the catabolite activating protein and the regulatory proteins of the *mal* and *ara* operons, in its ability to activate the initiation of transcription (reviewed in ref. 16). It differs from these other proteins in that its interconversion between active and inactive forms is mediated by covalent modification catalyzed by a second regulatory protein. This type of regulation is more complicated, but as has been pointed out in the case of enzymes such as glutamine synthetase and isocitrate dehydrogenase (15, 17), such control systems are exquisitely sensitive to changes in the environment.

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