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Complex Regulation of Urease Formation from the Two Promoters of the *ure* Operon of *Klebsiella pneumoniae*▽†

Qiong Liu and Robert A. Bender*

Department of Molecular, Cellular, and Developmental Biology, The University of Michigan, Ann Arbor, Michigan 48109-1048

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Klebsiella pneumoniae **can use urea as the sole source of nitrogen, thanks to a urease encoded by the *ureDABCEFG* operon. Expression of this operon is independent of urea and is regulated by the supply of nitrogen in the growth medium. When cells were growth rate limited for nitrogen, the specific activity of urease was about 70 times higher than that in cells grown under conditions of excess nitrogen. Much of this nitrogen regulation of urease formation depended on the nitrogen regulatory system acting through the nitrogen assimilation control protein, NAC. In a strain deleted for the *nac* gene, nitrogen limitation resulted in only a 7-fold increase in the specific activity of urease, in contrast to the 70-fold increase seen in that of the wild type. The *ure* operon was transcribed from two promoters. The proximal promoter (P1) had an absolute requirement for NAC; little or no transcription was seen in the absence of NAC. The distal promoter (P2) was independent of NAC, but its activity increased about threefold when the growth rate of the cells was limited by the nitrogen source. Transcriptional regulation of P1 and P2 accounted for most of the changes in urease activity seen under various nitrogen conditions. However, when transcription of *ureDABCEFG* was less than 20% of its maximum, the amount of active urease formed per transcript of *ure* decreased almost linearly with decreasing transcription. This may reflect a defect in the assembly of active urease and accounted for as much as a threefold activity difference under the conditions tested here. Thus, the *ure* operon was transcribed from a NAC-independent promoter (P2) and the most strongly NAC-dependent promoter known (P1). Most of the regulation of urease formation was transcriptional, but when *ure* transcription was low, assembly of active urease also was defective.**

Klebsiella pneumoniae is capable of using a large number of organic and inorganic compounds as sole sources of nitrogen. In almost every case, the production of the machinery for this assimilatory process is regulated by the general nitrogen regulatory (Ntr) system (13). In some cases, this regulation is direct and uses the phosphorylated form of NtrC to activate transcription from promoters that use σ^{54}-dependent RNA polymerase (13). In other cases, including the regulation of urease formation from the *ureDABCEFG* operon (2, 11), this regulation is mediated by the nitrogen assimilation control protein (NAC), which is itself under the control of NtrC phosphate (3, 11).

NAC has been shown to be an activator of transcription by the σ^{70}-dependent RNA polymerase, thus bringing σ^{70}-dependent operons under the control of the σ^{54}-dependent Ntr system (1). In the best-characterized example of NAC-mediated regulation, the histidine utilization (*hut*) operon, NAC binds to a site centered at −64 relative to the start of transcription and activates transcription independent of any small molecule or known modification (5). NAC also has been shown to activate from other positions, but in those cases the increase in transcription was less the 10-fold that seen at *hut* (7, 16, 17).

Macaluso et al. noted an apparent Ntr-independent regulation of urease formation in response to nitrogen limitation (11). At the time, this was the only known example of Ntr-independent nitrogen regulation, and this led us to explore the regulation of urease formation further.

The promoter region of the *ure* operon (*ureDp* region) was sequenced (8), and a NAC binding site in this region was identified both by gel mobility shift assay and by DNase I footprinting (5). NAC binds to a region that includes the sequence ATAA-N_5-TGNTAT, located 90 bp upstream of the start of *ureD* translation. If this sequence were centered at −64, then a possible match to the −10 consensus sequence (TATCTT) would provide an arrangement similar to that seen in the *hut* operon. In a preliminary study of the *ure* operon from the related species *K. oxytoca*, Collins and her coworkers (2) used primer extension to search for a transcriptional start site. The transcriptional start site that they reported was only 5 or 6 bp upstream of the start of *ureD* translation, and this start did not correspond to the putative −10 sequence identified by them by assuming that NAC bound at −64. In fact, the putative −10 sequence would have been located at −30 relative to the start of transcription. Since the DNA sequence of this region from *K. oxytoca* is the same as that from *K. pneumoniae*, it was unclear where the promoter lay, and it seemed likely that the start of transcription was farther upstream than the site identified by Collins et al. (2). Moreover, their primer extension suggested that the principal *ureD* promoter was about 10-fold regulated, whereas their assays of *ureDp-lacZ* operon fusions suggested that the regulation was 1,000-fold or more.

In their study, larger *ureDp* fragments, extending 0.7 kb farther upstream, showed considerable transcriptional activity even in the presence of excess ammonium, in which the Ntr system (and NAC) would be nonfunctional (2). It was not determined whether (or how) this activity was regulated. Thus,

* Corresponding author. Mailing address: Department of Molecular Cellular and Developmental Biology, The University of Michigan, Ann Arbor, MI 48109-1048. Phone: (734) 936-2539. Fax: (734) 647-0884. E-mail: rbender@umich.edu.

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TABLE 1. List of strains

Strain	Genotype	Function	Reference or source
KC2473	*hutC515 dad-1 nac306*::Tn*5tac1*	Source of *nac-306*::Tn*5tac1* (constitutive) allele	18
KC2653	*hutC515 dad-1* Δ[*bla*]*-2 str-6*	Nac^+ for assays	This laboratory
KC2668	*hutC515 dad-1* Δ[*bla*]*-2*		7
KB4921	*gltB200 glnL45 hutC*$^+$ *nac-1 zed-2*::Tn*5-131*	Source of *nac-1* (deletion) allele	6
KC6122	*hutC515 dad-1* Δ[*bla*]*-2 str-6 nac-1 zed-2*::Tn*5-131*	Nac^- for assays	P1 transduction
KC6146	*hutC515 dad-1* Δ[*bla*]*-2 str-6 nac306*::Tn*5tac1*	NAC^{Const} for assays	P1 transduction

there were several questions regarding the regulation of urease formation that seemed to suggest that there might be features unique to this NAC-regulated promoter. Where is the principle *ureD* promoter, and how strong is its regulation by NAC? Where is the upstream promoter, and is it regulated by nitrogen? Is expression from the upstream promoter sufficient to explain the Ntr-independent nitrogen regulation of urease formation?

MATERIALS AND METHODS

Strains and plasmids. All *K. pneumoniae* strains used in this study were derived from strain W70, which originally was called *K. aerogenes* (12). Strains used in this study are listed in Table 1. Isolation of single-copy *ureDp-lacZ* operon fusions was done by a two-step process. First, the fusions were generated in pRS415 (19) using standard methods involving primers, PCR, and cloning. These fusions next were transferred to a *pir*-dependent plasmid, pCB1583, which is derived from pKAS46 (20), to allow selection of integrants via the landing pad method, similar to that of Wu et al. (22). The specific details of the adaptation of this method to *K. pneumoniae* strain W70 will be described elsewhere (T. J. Goss and R. A. Bender, unpublished data). The transcriptional fusions then were integrated into strain KC2653 in single copies at the *rbs* locus.

Media. Minimal medium was W4 salts (W salts adjusted to an initial pH of 7.4) (11) supplemented with glucose (0.4%, wt/vol) as the sole carbon source. Nitrogen sources for nitrogen-limited growth were monosodium glutamate (0.2%, wt/vol), glutamine (0.1% or 0.04%, wt/vol; Calbiochem grade A, freshly made), or potassium nitrate (0.2%, wt/vol). A mixture of ammonium sulfate (0.2%, wt/vol) and glutamine (0.1%, wt/vol) was used as a nitrogen excess condition. Rich medium was L broth (15). When present, isopropyl-β-D-thiogalactopyranoside (IPTG) and 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside were added at final concentrations of 1 mM and 40 μg/ml, respectively. Ampicillin (100 μg/ml), kanamycin sulfate (50 μg/ml), and streptomycin (50 μg/ml) were added to the medium when appropriate. For cultures in which urease activity was measured, 1 μM nickel sulfate was included in the growth medium.

Genetic techniques. Recombinant DNA techniques were carried out as described by Maniatis et al. (14). Transduction was performed as described previously (4) using the phage P1vir.

Enzyme assays. Cultures were grown at 30°C to a density of about 50 Klett units. All assays were performed with cells that had been washed in 1% KCl and suspended at a concentration that contained 1 to 1.5 mg of protein per ml. Assays for β-galactosidase were performed on detergent-treated whole cells as described previously (11). Urease was assayed in the presence of 40 mM potassium phosphate, pH 7.5, 16 μg/ml cetyltrimethylammonium bromide, and 100 mM urea at 30°C. Reactions were initiated by the addition of urea. Portions of the assay mixtures were withdrawn at intervals, and the amount of ammonia released was determined by conversion to indolephenol (21), which was quantified by comparison to a standard curve and by measuring the absorbance at 546 nm. The protein content of whole cells was measured by the method of Lowry et al. (10) with bovine serum albumin as the standard. Specific activities are expressed as nanomoles of product formed (β-galactosidase) or substrate consumed (urease) per minute per milligram of cell protein at 30°C. All data are the results of three or more independent assays with standard errors of the means of less than 10% in every case.

Primer extension. Primer extension analysis was carried out as described by Yu and DiRita (23). Total RNA was isolated from DH5α/pCB1612 (for the full *ureDp* region), pCB634, or DH5α/pCB1630 (for UreP2) grown to mid-log phase in L broth. A template of 50 μg of total RNA was used in each reaction mixture. Two *ureD* open-reading-frame-specific primers, ure240 and ure200 (5′-CCGGC CTGGTGAAAGCGGAG-3′ and 5′-CCTGCCAGCCTTTTTTGAGTGGTGG TAAC-3′, respectively), were used for primer extension and for DNA sequencing. The DNA nucleotide sequence was determined for the primer extension by using the chain termination method with the Thermo sequenase radiolabeled terminator cycle sequencing kit (USB) supplied by the manufacturer.

RESULTS

Nitrogen regulation of urease expression: NAC-dependent and NAC-independent components. We cloned a short fragment containing 197 bp of upstream sequence and the first 64 bp of *ureD* and fused this fragment to a promoterless *lacZ* gene (see the sequence shown in Fig. 1). Using a landing pad technique similar to that described by Stewart et al. (22; Goss and Bender, unpublished), this operon fusion was transferred to the chromosome at the *rbs* locus of three *K. pneumoniae* strains. In the first strain (KC2653), the *nac* locus was wild type and NAC expression was driven by the native *nac* promoter and, thus, was regulated by the quality of the nitrogen source. In the second strain (KC6122), the *nac* locus was deleted and the strain produced no NAC at all. In the third strain (KC6146), the *nac* locus contained an insertion of Tn*5-tac* between the *nac* promoter and the *nac* coding sequence. In this strain, NAC expression was constitutive (NAC^{Const}) when IPTG was included in the growth medium. All three strains contained a wild-type *ure* operon at the normal chromosomal locus and a *ureDp-lacZ* fusion at the *rbs* locus, both in single copies.

As expected, urease formation in the Nac^+ strain was about 75-fold higher under conditions of nitrogen limitation than under nitrogen excess (Table 2). In the Nac^- strain, most of this regulation was lost, but the Nac^- strain still showed a sevenfold increase in urease activity under conditions of severe nitrogen limitation. This suggests that there is a NAC-independent activation of urease expression in response to nitrogen limitation. In the Nac^{Const} strain, urease activity was expressed at high levels even under nitrogen excess conditions; however, under nitrogen-limiting conditions the activity was about twofold higher than that under nitrogen excess, again suggesting that nitrogen limitation can increase urease expression even in the presence of NAC.

In order to determine whether this regulation of urease formation is at the transcriptional or posttranscriptional level, we next analyzed the expression of β-galactosidase from the *ure-lac* fusion in these strains. In the Nac^+ strain, β-galactosidase expression was about 12-fold higher under conditions of nitrogen limitation than under nitrogen excess. In the Nac^- strain, most of this regulation was lost, but the Nac^- strain still showed a two- to threefold increase in β-galactosidase expression under conditions of nitrogen limitation, again suggesting a NAC-independent expression of β-galactosidase from this pro-

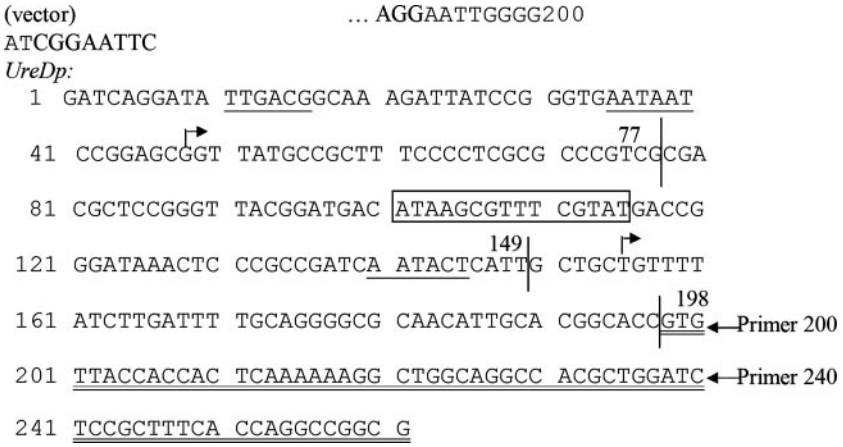

FIG. 1. Sequence of the *ureD* promoter region. Bent arrowheads indicate the start of transcription from the two promoters (P2 and P1, respectively) in the region. Underlined regions indicate the presumed −35 and −10 regions corresponding to P2. The boxed nucleotides indicate the NAC binding site. The double-underlined nucleotides are the beginning of the *ureD* coding region. The vertical bars with numbers indicate the endpoints of the deletions and fusions used. Primer 200 and Primer 240 indicate the positions of the 5′ end of the 29-mer and 20-mer primers, respectively, used in primer extension experiments.

moter region. In the Nac^{Const} strain, β-galactosidase was expressed at high levels even under nitrogen excess conditions; however, under nitrogen-limiting conditions it was somewhat higher than that under nitrogen excess, again suggesting that nitrogen limitation can increase expression from this promoter even in the presence of NAC.

In other words, the pattern of β-galactosidase regulation was qualitatively similar to the pattern of urease regulation. However, there were significant quantitative differences. For example, in the Nac$^+$ strain, urease expression was 75-fold higher under nitrogen-limiting conditions than under excess conditions, whereas β-galactosidase expression was only 12-fold higher. Since there appeared to be both a NAC-dependent and a NAC-independent regulation affecting expression, we suspected that there might be more than one promoter in this region, as was found for *K. oxytoca* (2). We therefore generated a deletion fragment (Fig. 2, P1) that lacked 77 bp of the upstream region. This fragment retains the NAC binding site and all the material between the NAC binding site and the start of the *ureD* coding region. In the Nac$^+$ strain there was a very strong NAC-dependent regulation of β-galactosidase expression, with at least 370 times more β-galactosidase formed under nitrogen limitation than under nitrogen excess. All of this regulation was NAC dependent, since there was no detectable β-galactosidase expression in the Nac$^-$ strain. In the Nac^{Const} strain, β-galactosidase expression was constitutive at high levels, and there was no increase under nitrogen limitation.

A NAC-independent urease promoter, *ureDp*$_2$. In an attempt to identify the NAC-independent promoter, we generated another fusion (Fig. 2, P2) that contained only the 77 bp of the upstream region fused to the *ureD* coding sequence and *lacZ*. β-Galactosidase was expressed at high levels from P2 in all three strains, and this expression was independent of NAC. However, there was a two- to threefold increase in β-galactosidase expression under nitrogen-limiting conditions not only in the Nac$^+$ strain but also in the Nac$^-$ strain and the Nac^{Const} strain. Thus, there appears to be two promoters that drive *ure* operon expression, an operon-proximal promoter, P1, that is very strongly dependent on NAC, and an operon-distal one,

TABLE 2. NAC dependence of urease activity and *ure-lac* expression under nitrogen limitation and nitrogen excess growth conditions

Strain[a]	Nitrogen condition[b]	Urease (sp act)[c]	β-Gal (sp act)[d]					
			Full	P1	P2	P1Δ	P2 + 30 bp	Full + 253 bp
Nac$^+$	Limiting	1,486	5,976	3,724	5,412	4,737	2,219	4,607
	Excess	19.1	511	<10	1,526	<10	680	330
Nac$^-$	Limiting	160	1,310	<10	4,081	<10	1,743	814
	Excess	23.8	538	<10	1,448	<10	577	378
Nac^{const}	Limiting	1,377	5,287	2,935	4,778	4,390	2,069	4,252
	Excess	606	3,795	2,724	2,189	5,770	1,065	3,144

[a] Nac$^+$, Nac$^-$, and Nac^{Const} strains were KC2653, KC6122, and KC6146, respectively. IPTG was included in the growth medium of KC6146 to induce constitutively high *nac* expression.

[b] Cells were grown in glucose minimal medium under severe nitrogen limitation (glutamate as the sole nitrogen source) or nitrogen excess (a mixture of ammonium sulfate and glutamine) as described in Materials and Methods.

[c] Specific activity of the urease formed in cells from the wild-type *ureDABCEFG* operon.

[d] Specific activity of β-galactosidase (β-gal) expressed from a *ureD-lacZ* fusion inserted into the *rbs* locus. The structures of the promoters in these fusions (Full, P1, P2, etc.) are depicted in Fig. 2. The β-galactosidase expressed from the endogenous *K. pneumoniae lac* operons was determined in isogenic strains lacking the *ureD-lacZ* fusion and was subtracted from the values reported. That amount was quite low (less than 20 U/mg in the presence of IPTG and less than 2 U/mg in the absence of IPTG).

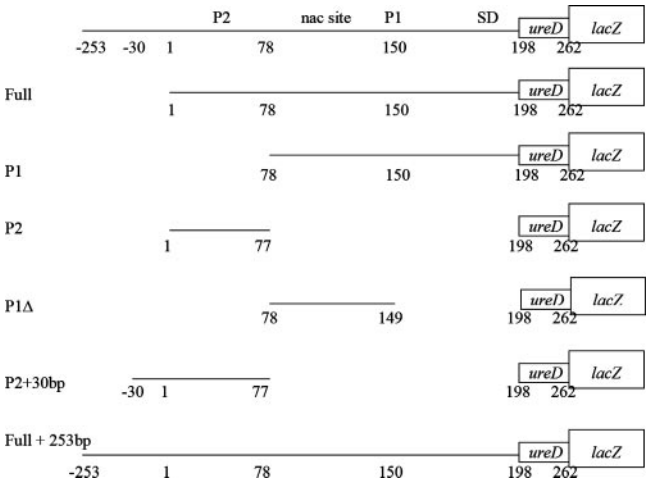

FIG. 2. The *ureD-lacZ* transcriptional fusions used. Solid lines indicate DNA sequences present, and the numbers on the lines indicate the last nucleotide remaining, using the numbering of Fig. 1. P1 and P2 indicate the approximate positions of the two promoters, and SD indicates the approximate position of the Shine-Dalgarno sequence in front of the *ureD* coding sequence. Full refers to the 262-bp fragment illustrated in Fig. 1. The negative numbers indicate constructs that contain *K. pneumoniae* DNA upstream of the sequence shown in Fig. 1. *lacZ* indicates the promoterless *lacZ* sequence in plasmid pRS415 (19).

P2, that is independent of NAC but still responds to nitrogen limitation by an unknown mechanism.

If the activities of the two promoters were independent of each other, we would expect that β-galactosidase expression from the full-length promoter should be the sum of the expression from the two promoters in isolation. This clearly was not the case. For example, in the Nac$^-$ strain, all expression comes from P2 (P1 is inactive in the absence of NAC). However, the isolated P2 promoter gave significantly higher levels of β-galactosidase than did the full promoter (Table 2). One explanation for this discrepancy might be that the long leader between P2 and the start of translation might be responsible for the reduction in expression. To test this, we examined a variant of the P1 construct (P1Δ) (Fig. 1) in which 50 bp of sequence between P1 and the start of translation had been deleted. In this construct, only NAC-dependent transcription should yield β-galactosidase. Again, expression of β-galactosidase was significantly higher in the deleted version than when the intervening sequence was present. Therefore, our working model is that some feature of the sequence between the promoters and the coding sequence is responsible for attenuating the effectiveness of the transcription.

The data in Table 2 suggest that there is a NAC-independent promoter within the 77-bp region present in construct P2 and a NAC-dependent promoter located in the 72-bp region between bp 78 and 149. To determine their locations precisely, we next carried out a primer extension analysis to identify the start of transcription in these regions. The data in Fig. 3 suggest that the transcript coming from P2 begins with the G at bp 48, and the transcript from P1 begins at bp 155 or 156 (T and G, respectively). An analysis of the sequence upstream of bp 48 reveals the sequence AATAAT about 10 bp upstream of the start of transcription (of P2), a reasonable match to the consensus −10 region of σ^{70}-dependent promoters. Eighteen base pairs upstream of that putative −10 region we found the sequence TTGACG, a good match to the consensus −35 region for σ^{70}-dependent promoters. We were concerned that the sequence of the promoter extends only to −47 in our constructs and that we might be missing regulatory information upstream of P2. However, similar constructs with an additional 30 or 253 bp of upstream sequence gave the same pattern of transcription (data not shown).

Upstream from the P1 transcription start site, we found no sequences resembling either a −10 or −35 consensus sequence for σ^{70}-dependent promoters. At best, we noted a sequence, AATCAT, which might represent a −10 consensus sequence, but this was located at −13, a bit farther from the start of transcription than would be expected. The absence of an obvious promoter consensus for P1 is not surprising because, in the absence of NAC, no expression of the promoter was detectable.

Posttranscriptional effects on urease activity. If there were no posttranscriptional effects, the ratio of urease to β-galactosidase (enzyme activity to transcriptional activity) should be constant, at least for a given construct. However, the data in Table 2 contradict this assumption. For example, if we compare the ratio of enzymatic activity from the chromosomally encoded *ure* operon to transcriptional activity from the full-length fusion, we find that the ratio varies from a low of 0.04 to a high of 0.26. Two features became immediately obvious from the data in Table 2. First, even under conditions in which only P2 was expressed (the Nac$^-$ strain), the ratio of urease to β-galactosidase was not constant. Second, in general, the lower the amount of transcription, the lower the ratio of urease to β-galactosidase. Therefore, we examined this result in more detail.

A wild-type strain carrying the full-length *ure-lac* fusion (to measure transcription) and the wild-type *ure* operon was grown with three different *nac* alleles in a variety of nitrogen sources, as described in Materials and Methods, to give a range of transcriptional activities for the *ure* promoters (Fig. 4). The results of these 20 experiments show clearly that, below 2,000 U of transcription (measured as β-galactosidase), the enzyme activity per transcript (measured as the urease/β-galactosidase ratio) fell almost linearly with transcriptional activity. At amounts of transcription corresponding to 2,000 U of β-galactosidase or more, the value of urease/β-galactosidase more or less leveled off, though the actual value of that ratio was slightly dependent on the growth medium, with the most nitrogen-limited cultures giving the highest ratios (above 0.2). The raw data for this figure are given in Table S1 in the supplemental material. There was considerable scatter in the ratios for cells with high levels of transcriptional activity, but the linearity of the data at lower levels of transcription was clear.

DISCUSSION

Collins et al. (2) showed that $ureDp_1$ is activated as much as 1,000-fold by nitrogen starvation. We show here that this regulation is mediated entirely by NAC. Since there was no detectable transcription from $ureDp_1$ in the absence of NAC, it was not surprising that we were unable to identify a good

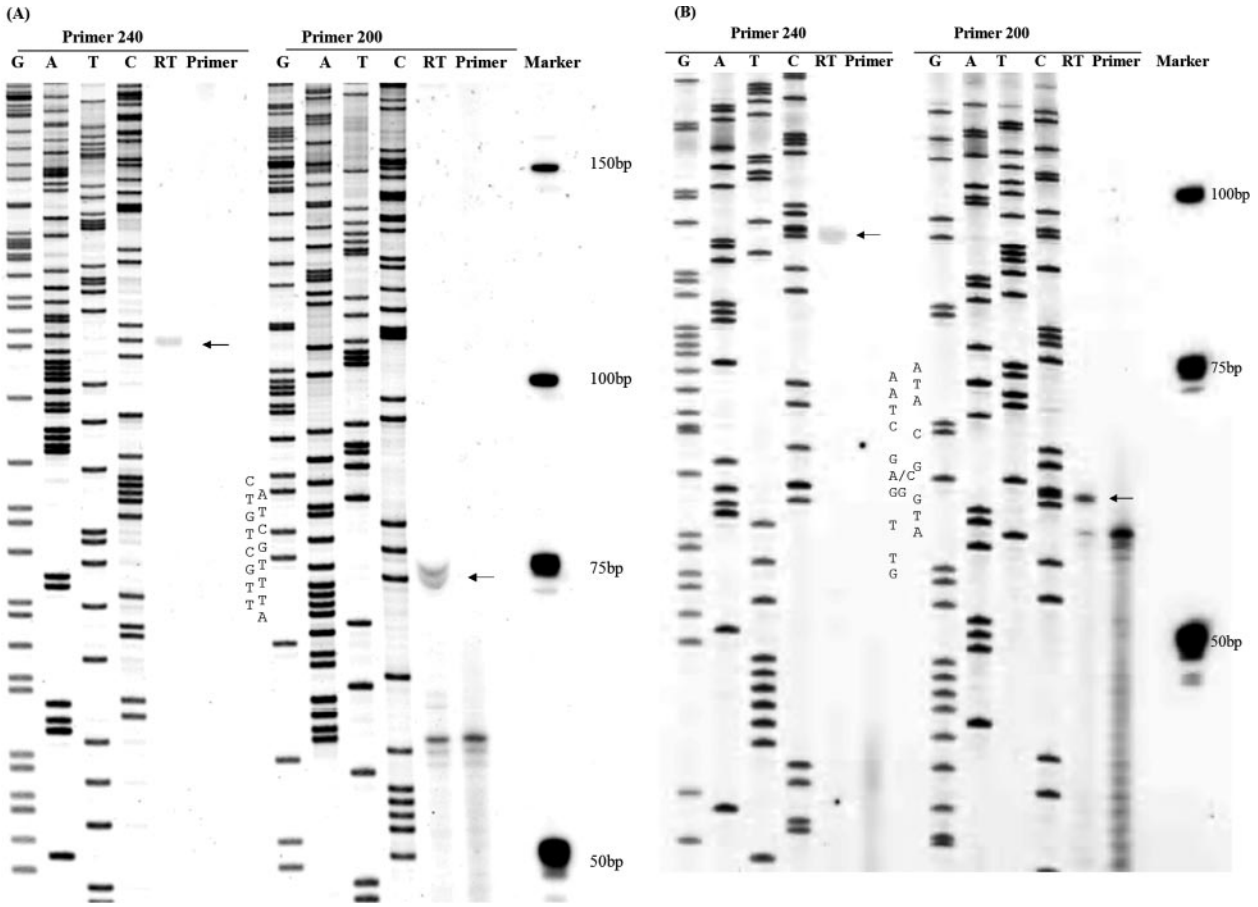

FIG. 3. Primer extension of mRNA from the *ureD* promoter region. Primers extending back to bp 200 and 240 were used to determine the start of transcription. The same primers were used to generate a DNA sequencing ladder. Arrows indicate the start of transcription, and the DNA sequence (complementary to the sequencing ladder but matching the sequence shown in Fig. 1) is indicated. GATC indicates the DNA sequencing ladder. RT indicates the experimental lane with reverse transcriptase present during the reaction. Primer indicates a control reaction with no reverse transcriptase. Note that Primer 200 gave an artifactual signal, somewhat shorter than the actual runoff transcript generated by reverse transcriptase. (A) Primer extension of mRNA isolated from strain DH5α carrying a plasmid with the complete *ureDp* region (designated Full in Fig. 2) and a plasmid expressing *K. pneumoniae nac* constitutively. (B) Primer extension of mRNA from DH5α carrying only a plasmid with $ureDp_2$ (designated P2 in Fig. 2).

match to the consensus for −10 or −35 regions. The best we found was a sequence, AATACT, located at −13 rather than −10. Whatever the actual promoter sequence may be, its absolute dependence on NAC is unusual. For all other promoters studied to date, NAC activates expression only 3- to 10-fold, reflecting the fact that those promoters had substantial basal expression in the absence of positive regulators. The $ureDp_1$ promoter also is atypical in the relative positions of RNA polymerase and NAC. When a NAC binding site was placed in front of the *lacZ* promoter, it activated transcription strongly (10-fold) when the site was centered at −64 and weakly when it was centered at −42, −52, and −54. It was not able to activate transcription when it was positioned at −47, −49, −59, −69, or −74 (17). Thus, the activation from a site in $ureDp_1$ at −47 was unexpected both for its strength and its location.

The earlier work with *K. oxytoca* (2) strongly suggested the existence of an Ntr-independent promoter for *ureD*, but its location and regulation were not identified. The primer extension data that identify the *ureP2* transcription start site at bp 48 agree well with the presence of a rather good match to a consensus σ^{70}-dependent promoter (TTGACG-N_{18}-AATAAT; located at bp 10 to 40 in Fig. 1). Growth rate limitation by poor nitrogen sources results in a slight (two- to threefold) increase in $ureDp_2$ activity, but our data define only the quantity of the effect (and the fact that it increases with increasing limitation, up to the limit of two- to threefold), not the molecular mechanism. In other words, we are unable to distinguish among models such as growth rate control, stringent response (caused by nitrogen starvation), or a variety of other alternatives. In any event, this transcriptional effect on $ureDp_2$ accounts for only a portion of the Ntr-independent nitrogen regulation of urease formation.

Another portion of that regulation is an indirect effect of the observation that, when transcription of the *ure* operon is low, the amount of active urease formed per transcript is low. Again, our data define the magnitude of the effect (the amount of active urease formed per transcript is at least sevenfold lower when transcription is 8% of the maximum) but do not address the molecular mechanism of the effect. The *ure* operon encodes not only the structural genes for the enzyme (*ureABC*)

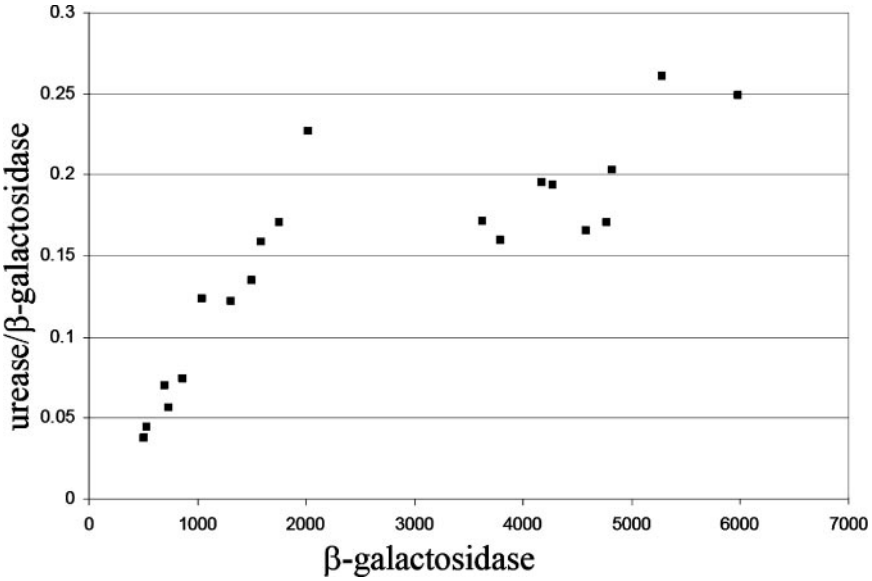

FIG. 4. Amount of urease activity formed per transcript as a function of transcription amount. Three strains differing only in their *nac* allele were used, KC2653 (Nac^+), KC6122 (Nac^-), and KC6146 (Nac^{const}) (which was grown both with and without IPTG present), and each carried a wild-type *ure* operon and a *ure-lacZ* fusion (designated Full in Fig. 2) inserted at the *rbs* locus. The strains were grown in glucose minimal medium with five different nitrogen sources, resulting in a variety of levels of *ure* transcription. The specific activity of the urease formed by the cells was determined by direct assay of urease, and the amount of transcription from the *ure* promoter was determined indirectly by measuring β-galactosidase from the *ure-lac* fusion. The ratio of urease activity to β-galactosidase activity (urease activity per transcript) was plotted against β-galactosidase activity (amount of transcription). The raw data for the 20 different conditions are given in Table S1 in the supplemental material. All data were the result of three or more assays of independent cultures with standard errors of the means of less than 10% in all cases.

but also the genes required for nickel incorporation, *ureD* and *ureEFG* (21). It is tempting to speculate that it is a limitation for the nickel-incorporating apparatus that restricts the amount of active urease that can be formed. However, our data also are consistent with the hypothesis that at low concentrations of UreA, UreB, and UreC subunits, apoenzyme assembly is defective (9).

What is clear is that the two- to threefold activation of $ureDp_2$ in response to nitrogen limitation is not mediated by NAC or the Ntr system. Strains lacking NAC (Table 2) or NtrC (data not shown) both show the elevation in response to growth-rate-limiting nitrogen sources. However, $ureDp_1$ remains the focus of our future work. Its absolute dependence on NAC for expression is unique, and the position of the NAC binding site relative to the polymerase suggests a very different mechanism for the activation of $ureDp_1$ than for *hutP*.

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