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Nitrogen regulation of transport operons: Analysis of promoters *argTr* and *dhuA*

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Summary. In *Salmonella typhimurium* the periplasmic permeases for histidine and for lysine-arginine-ornithine are regulated by nitrogen availability. The nature of the *dhuA* and *argTr* promoters of the operons coding for these permeases was analyzed by placing the galactokinase gene under their control (in vector pKO-1). *argTr* was found to respond to nitrogen regulation. We investigated the involvement of a mirror symmetry in *argTr* in its regulation by nitrogen. It had been postulated previously (Higgins and Ames 1982) that mirror symmetries might act as protein recognition sites important in regulation of gene expression. Here we demonstrate that the mirror symmetry in *argTr* is not involved in nitrogen control. Contrary to expectation, the *galK* gene was not regulated by nitrogen when it was placed under *dhuA* control. Here we propose a possible explanation for this finding.

Key words: Nitrogen regulation – Mirror symmetry – Periplasmic permease – Promoters

The high-affinity histidine permease of *Salmonella typhimurium* consists of four proteins, the periplasmic histidine-binding protein J and the membrane proteins Q, M, and P encoded by an operon located at 48.5 min on the *S. typhimurium* chromosomal map (Ames et al. 1977; Ames 1985; Higgins et al. 1982). Preceding the structural genes is the regulatory region *dhuA* (Ames et al. 1977). Contiguous to this operon is the structural gene (*argT*) for another periplasmic binding protein that binds lysine, arginine, and ornithine (LAO) together with its regulatory locus, *argTr* (Higgins and Ames 1982). All the structural genes are subject to nitrogen control as indicated by elevated levels of the respective proteins after growth on a poor nitrogen source (Kustu et al. 1979). Characterization of the response to nitrogen limitation was also performed by utilizing Mu*d1* (Ap, *lac, cts*) operon fusions (Stern et al. 1984). Regulation of expression was shown to be mediated by the global nitrogen control system *ntr*, consisting of the gene products *ntrA*, *ntrB*, and *ntrC* (Ames and Nikaido 1985; Kustu et al. 1979). The entire nucleotide sequence of the two operons and their regulatory region has been determined (Higgins and Ames 1982; Higgins et al. 1982). We have demonstrated that the nitrogen regulatory protein NtrC, product of gene *ntrC* (*glnG* in *Escherichia coli*) binds to a specific sequence in the *dhuA* promoter region (Ames and Nikaido 1985). In order to get further information about these promoter regions and the sequences specifically responsible for nitrogen regulation, we cloned each regulatory region, *argTr* and *dhuA*, into plasmid pKO-1, a vector especially developed for the study of prokaryotic regulatory signals (McKenney et al. 1981). Three interesting features that might be involved in regulation had been identified in both regulatory regions (Higgins and Ames 1982): several dyad symmetries, a region of specific homology, and an alphabetic mirror symmetry (as defined in Higgins and Ames 1982, e.g., GACTNNNTCAG). We turned our attention first to the significance of the mirror symmetry. This sequence was hypothesized as being involved in regulation, since an homologous mirror symmetry was also found in the regulatory region of the glutamine synthetase gene (*glnA*), another gene under nitrogen control. Mirror symmetries were also found in the promoter regions of several operons, thus suggesting that they may serve important functions. These mirror symmetries are not true symmetries because of the polarity of the sugar-phosphate backbone. However, preliminary model building by cylindrical projection (S.-K. Kim, unpublished data) indicated that they constitute a twofold symmetry of functional groups (hydrogen bond donors and acceptors) in the minor groove of DNA (Higgins and Ames 1982). Such a symmetrical structure might therefore serve as a recognition site for a dimeric protein. In this study we specifically mutagenized the mirror symmetry in *argTr* using synthetic oligonucleotides, to test whether it plays a role in nitrogen control. Our results show that the mirror sequence is not involved in nitrogen regulation. We also present evidence that nitrogen regulation at *dhuA* may be due to a mechanism other than through classic activation by the NtrC protein at the promoter site proper.

Plasmid derivatives containing each promoter region, *dhuA* and *argTr*, controlling transcription of *galK* were constructed using pFA9 as a source of DNA. According to the scheme shown in Fig. 1, pFA9 is a pBR322 derivative carrying the *argTr*, *argT*, and *dhuA* regions (Ames and Nikaido 1985; Higgins and Ames 1982). Plasmid DNA was isolated as described (Clewell and Helinski 1969) with an additional phenol extraction step and final purification in a CsCl density gradient. Plasmid pKO-1 (McKenney et al. 1981) was digested with *Sma*I and subsequently dephosphorylated with alkaline phosphatase from *E. coli* at 65° C. The *argTr* and *dhuA* fragments (530 bp and 326 bp respectively) were obtained by digesting pFA9 with *Hae*III and

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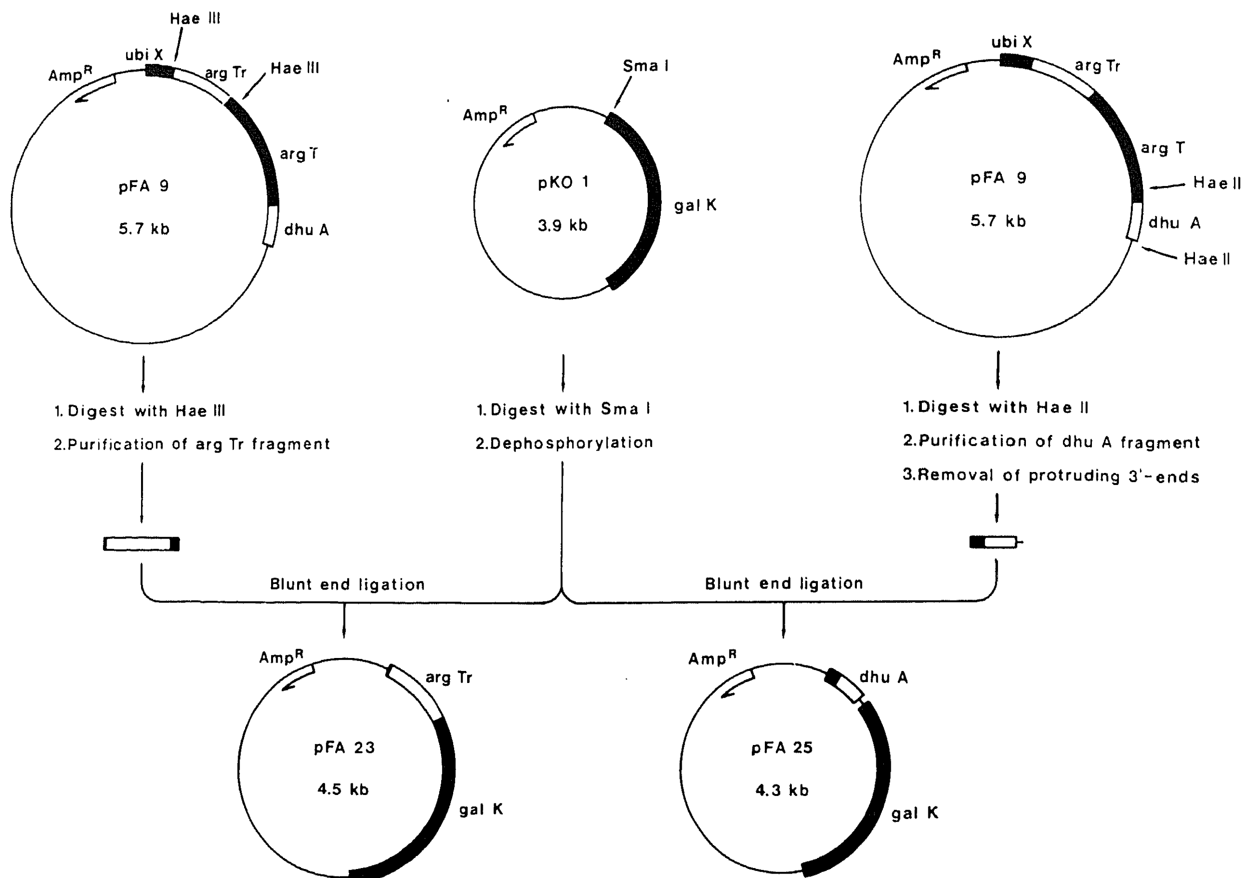

Fig. 1. Construction of promoter-galactokinase vectors. The plasmids, fragments, and relative positions of the restriction sites are drawn to scale

*Hae*II respectively, and isolating the fragments from a polyacrylamide gel by electroelution. Protruding 3′ ends were removed from the *dhuA* fragment with T4 DNA polymerase (Maniatis et al. 1982). Vector and fragments were mixed (10 times molar excess of fragment over vector), blunt-end ligated at room temperature for 3 h with T4 DNA ligase, transformed into competent cells of *E. coli* N100, a *galK recA* mutant (McKenney et al. 1981), and plated onto the following media: nutrient broth supplemented with 50 µg/ml ampicillin, and a minimal medium (Gutnick et al. 1969) containing 0.4% galactose, 10 mM sodium glutamate, 50 µg/ml proline, and 50 µg/ml ampicillin. In the case of the *argTr* promoter, 40 transformants were picked from the minimal medium and nutrient broth plates and screened by small-scale preparations of plasmid DNA (Maniatis et al. 1982) and digestion with restriction endonuclease *Taq*I. Only one clone was found to contain the desired fragment and orientation by restriction endonuclease analysis with *Taq*I, *Hin*cII, and *Hin*fI (data not shown). This plasmid was named pFA23. In the case of the *dhuA* promoter, 2 out of 24 transformants were shown to contain the desired construction by digestion with *Hae*III, *Taq*I, and *Bgl*I (data not shown). The *dhuA*-containing plasmid was named pFA25.

Since the promoter activities were not easy to monitor in *E. coli* N100 on several indicator plates (for unknown reasons), we transformed the plasmids into *E. coli* C600K, a *galK leu thr* auxotroph (McKenney et al. 1981). Transformants carrying either pFA23 or pFA25 gave red colonies on MacConkey/galactose/ampicillin plates after growth for 24 h at 30° C. Transformants with pKO-1 (i.e., no promoter insert) were white after 24 h but turned red when further incubated, probably due to the basal galactokinase expression (McKenney et al. 1981). Table 1 shows the galactokinase activity of these strains: both plasmids pFA23 and pFA25 exhibited promoter activity. Basal gene expression under N-excess was approximately twofold higher from

Table 1. Response of transport promoters to nitrogen regulation

Plasmid[a]	Promoter	Relative galactokinase expression[b]		Induction ratio
		N-rich	N-poor	N-poor/N-rich
pFA23	*argTr*[c]	100	430	4.3 (5.1)[f]
pFA25	*dhuA*[d]	219	260	1.2 (1.2)
pKO-1	No[e]	19	19	1.0

[a] All plasmids were present in *Escherichia coli* C600K, grown in minimal medium (Gutnick et al. 1969) supplemented with 0.3 mM threonine, 0.3 mM leucine, 25 µg/ml ampicillin, 0.4% glucose, and either 10 mM glutamate (N-poor) or 10 mM NH_4Cl (N-rich)
[b] Galactokinase activity was measured as described (McKenney et al. 1981) and is expressed in terms of percentages of *argTr* under nitrogen-rich conditions
[c,d,e] Average values of 18, 8, and 6 assays, respectively
[f] Numbers in parentheses are the induction ratios calculated after subtraction of the pKO-1 level

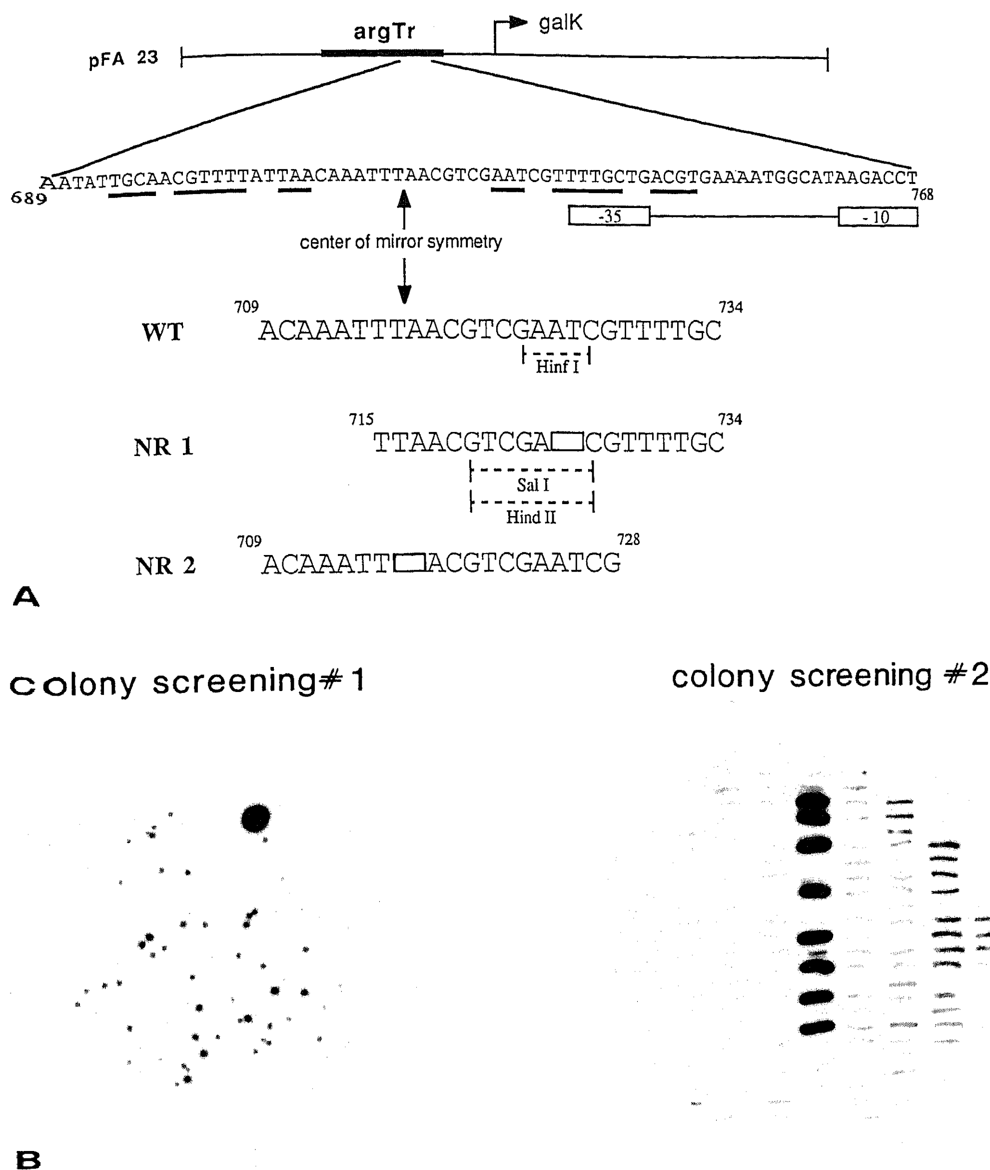

Fig. 2A, B. Construction of mutations in the *argTr* promoter region. **A** The sequences of the wild type and of oligonucleotides NR1 and NR2 are shown. Symbols are as follows: *thick line, argTr* insert in pFA23; *thin line,* pKO sequence in pFA23; *underlined* nucleotides, mirror symmetry; *arrows,* center of mirror symmetry; *open bar joined by a line,* postulated *argTr* promoter; *open bars,* 2 bp deletions caused in NR1 or NR2; *dashed lines,* relevant restriction sites. Base pair numbers are as in Higgins and Ames (1982). **B** Screening for mutations by colony hybridization with ^{32}P-labeled oligodeoxynucleotide NR1. Putative mutants (dark spots in the lefthand autoradiogram) were purified and screened a second time by hybridization (righthand autoradiogram). Each vertical row contains streaks obtained from transformants of one plasmid type: first two left lines, wild-type plasmid; center line, darkest colony from the first colony screening for NR1 mutagenesis (lefthand autoradiogram); all others, light colonies from the first colony screening

dhuA than from *argTr*. The *argTr* promoter showed on average 5.1-fold stimulation under nitrogen limitation. The results obtained with *argTr* are in agreement with the data obtained previously with Mu*d1* (Ap, *lac, cts*) fusions (Stern et al. 1984). These data indicate that regulation at *argTr* is measurable in this multicopy vector system. Transcription from the unmodified pKO-1 vector was relatively high, on average 19% of that measured for *argTr* under N-excess. We excluded the possibility that the observed nitrogen regulation at *argTr* was due to a copy number effect by determining the amount of plasmid DNA in cells containing pKO-1 and pFA23 (Crosa and Falkow 1981). On average, 50 plasmid copies were found under both N-rich and N-poor growth. Similar values have been reported for other pKO-1 constructions (Duester et al. 1982).

Figure 2A is a schematic representation of the *argTr* promoter region and some of its features of interest. The mirror symmetry is located between bp 694 and 741 and overlaps partially the -35 region of the postulated *argTr* promoter (Higgins and Ames 1982). To test whether the mirror symmetry is involved in nitrogen control, we specifically mutagenized this feature in pFA23 by oligonucleotide-directed mutagenesis. Two different oligodeoxynucleotides (NR1 and NR2, 18 bp each) were utilized (Fig. 2A). Each contains a 2 bp deletion. Oligonucleotide NR1 has the deletion downstream of the center (bp 725 and 726), and oligo-

nucleotide NR2 has the deletion in the center of the mirror symmetry (bp 716 and 717). NR1 changes the sequence of one arm of the symmetry, while NR2 displaces one arm of the symmetry relative to the other, along the DNA helix. Therefore, either mutation should interfere with protein binding if this sequence functions as a regulatory protein-binding site in nitrogen control. Mutagenesis was carried out according to Dalbadie-McFarland et al. (1982). Double-stranded circular plasmid DNA (10 µg of pFA23) was nicked in one strand with *Nar*I (New England Biolabs), which acts with low efficiency on a single site located inside the *galK* gene at position 563, approximately 700 bp downstream of the *argTr* promoter region. The nicked DNA was rendered only partially single stranded, using 15 units of exonuclease III at 37° C for 30 min. In vitro DNA synthesis was primed by the synthetic oligonucleotides NR1 and NR2, respectively (2000-fold excess of primer over template). The oligonucleotides were synthesized manually on silica support (Urdea et al. 1983), but N,N-diisopropylphosphoramidites (McBride and Caruthers 1983; Adams et al. 1983) rather than the N,N-dimethylanalogs were utilized. The resulting heteroduplex plasmids were transformed into *E. coli* C600K. Transformants were screened for the presence of mutations by colony hybridization with ^{32}P-labeled oligodeoxynucleotides (NR1 and NR2 probes) (Fig. 2B; only the NR1 mutagenesis is shown. Similar results were obtained with NR2). Since transformants with mixed and pure genotype were expected after the first transformation and hybridization, putative mutants (dark spots in the lefthand autoradiogram) were purified and screened a second time by hybridization (righthand autoradiogram). Cells containing the NR1 mutation can be clearly identified as being much darker. Increasing the wash temperature to 40° C increased specificity (data not shown). Three out of 7 mutants had a mixed genotype. Mutagenesis with NR1 yielded 1 mutant (pFA32) out of 2188 cells tested (0.046%), while mutagenesis with NR2 resulted in 6 mutants (pFA33 was saved) out of 1840 transformants, i.e., about 7 times higher (0.32%). The mutation frequency lay in the expected range (Dalbadie-McFarland et al. 1982). The presence of the NR1 mutation was confirmed by restriction analysis. As predicted from the DNA sequence (Fig. 2A), the 2 bp deletion present in NR1 created two new restriction sites: a *Sal*I site in position 720 and a *Hin*dII site in position 719. It also destroyed the *Hin*fI site in position 723 (data not shown). The 2 bp deletion caused by the NR2 oligonucleotide could not be checked by restriction analysis because sites were neither created nor destroyed. Since the phenotype of both mutants was confirmed by deletion mutagenesis (see below), no further characterization of the mutant plasmids was carried out.

The effect of nitrogen limitation on the mutant promoters was measured by *galK* expression under nitrogen-rich and nitrogen-poor conditions. Table 2 shows that the basal *galK* expression is not affected (NR2 mutation in pFA33) or only slightly (NR1 mutation in pFA32), as compared with the wild-type pFA23. Thus, the 2 bp deletion, 15 bp upstream of the postulated -35 region in pFA33 had no effect on the promoter strength, whereas the NR1 mutant, having the 2 bp deletion only five bases upstream of the -35 region, had a slight effect. In the latter case, it might be that the NR1 mutation has affected the RNA polymerase binding site. Interestingly, both mutants are still regulated normally by nitrogen availability: the induction from pFA32 and pFA33 was 5.1-fold and 4.5-fold, respectively, and thus comparable with the 5.1-fold induction from wild-type pFA23. This implies that the mirror symmetry in *argTr* is not involved in nitrogen control. To confirm the data we deleted all *argTr* sequences upstream of the newly created *Sal*I site at bp 720 in pFA32 (see NR1 in Fig. 2). The deletion which included most of the mirror symmetry, including alterations introduced by oligonucleotide-directed mutagenesis, had no effect on nitrogen regulation (data not shown). In agreement with this result, additional data (which will be published elsewhere) indicate that all sequences responsible for nitrogen control are located downstream of bp 720.

Table 2. Response of mutants with deletions in the mirror symmetry of *argTr* to nitrogen regulation

Plasmid	Genotype	Relative galactokinase[a] expression		Induction ratio
		N-rich	N-poor	N-poor/N-rich
pFA23	WT	100	510	5.1
pFA32	NR1	63	326	5.1
pFA33	NR2	113	506	4.5

[a] Corrected for pKO-1 background value

In conclusion, our results strongly indicate that the mirror symmetry is not involved in nitrogen control at *argTr*. This does not exclude the possibility that mirror symmetries are involved in regulation at other promoters.

In attempting to define the features of *dhuA* which are involved in nitrogen regulation, we analyzed the effect of nitrogen starvation on the *dhuA*-containing *galK* vector, pFA25. Contrary to our expectations (Kustu et al. 1979; Stern et al. 1984), the cloned *dhuA* promoter does not respond to nitrogen starvation (Table 1). This unexpected result could be explained if regulation at *dhuA* occurred via anti-termination of transcripts initiated in the upstream operon: within *argTr* and *argT*. In this case increased expression of the chromosomal histidine transport operon would be caused by transcriptional read-through. In plasmid pFA25 no *dhuA* regulation by nitrogen would occur, because the *argTr* promoter is missing. In agreement with this hypothesis, the binding site for the nitrogen regulatory protein NtrC in the *dhuA* promoter overlaps a rho-independent termination site for nitrogen-regulated transcripts initiating upstream (Ames and Nikaido 1985). Preliminary evidence indicates that there is indeed anti-termination within *dhuA* (K. Storm and G. Ames, unpublished data). This would be an unusual and interesting property of a protein normally acting as an activator. In view of the fact that the NtrC protein binding sites have been implicated in an enhancer-like function in nitrogen regulation (Reitzer and Magasanik 1986) the possibility will have to be considered that the binding in *dhuA* is affecting transcription at the upstream *argTr* promoter and that it affects expression from *dhuA* only secondarily.

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