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Regulatory regions of two transport operons under nitrogen control: Nucleotide sequences

(histidine and arginine transport/*Salmonella typhimurium*/promoter sequences/protein–DNA interactions/mirror symmetry)

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ABSTRACT **We have determined the nucleotide sequences of the regulatory regions from two amino acid transport operons from *Salmonella typhimurium*: *dhuA*, which regulates the histidine transport operon, and *argTr*, which regulates *argT*, the gene encoding the lysine–arginine–ornithine-binding protein, LAO. The promoter for the histidine transport operon has been identified from the sequence change in the promoter-up mutation *dhuA1*. Neither regulatory region has any of the features typical of the regulatory regions of the amino acid biosynthetic operons, indicating that regulation of at least these transport genes does not involve a transcription attenuation mechanism. We have identified three interesting features, present in both of these sequences, which may be of importance in the regulation of these and other operons: a "stem–loop–foot" structure, a region of specific homology, and a mirror symmetry. The region of mirror symmetry may be a protein recognition site important in regulating expression of these and other operons in response to nitrogen availability. Mirror symmetry as a structure for DNA–protein interaction sites has not been proposed previously.**

Regulation of the active transport of amino acids in bacteria is poorly understood at a molecular level, despite many empirical observations that transport activity can vary markedly under different growth conditions. Expression of the high-affinity histidine transport operon of *Salmonella typhimurium* seems to be regulated independently from the histidine biosynthetic operon (unpublished observations). Thus, it seems that regulation of at least this amino acid transport operon involves regulatory mechanisms other than the transcription attenuation mechanism described in detail for several amino acid biosynthetic operons (1–3). On the other hand, it has been suggested that regulation of the leucine–isoleucine–valine- and the leucine-specific transport systems of *Escherichia coli* might be regulated by such an attenuation mechanism (4). In order to clarify the mechanisms by which amino acid transport operons are regulated, we have studied two transport operons in detail: one encoding the histidine transport proteins, the other encoding the lysine–arginine–ornithine-binding protein (LAO).

The high-affinity histidine transport system of *S. typhimurium* has been characterized in considerable detail. Four genes are required for transport, *hisJ*, *hisQ*, *hisM*, and *hisP*, which encode, respectively, a periplasmic histidine-binding protein, J, a membrane protein, Q, a protein of as yet unknown location, M, and an inner membrane protein, P (refs. 5 and 6; unpublished data). Together with a genetically defined regulatory locus, *dhuA*, these genes form an operon located at 48.5 minutes on the recalibrated *S. typhimurium* chromosomal map (ref. 7; Fig. 1). The membrane-bound P protein and the periplasmic histidine-binding protein, J, interact with each other during transport (8). The P protein also interacts with another periplasmic binding protein, LAO (the lysine–arginine–ornithine-binding protein), which is involved in arginine uptake (9). The LAO protein is encoded by the *argT* gene, which maps adjacent to the histidine transport operon (Fig. 1). The *argT* and *hisJ* genes are closely related: they are more than 70% homologous and are believed to have arisen from a single ancestral gene by duplication and divergence (10).

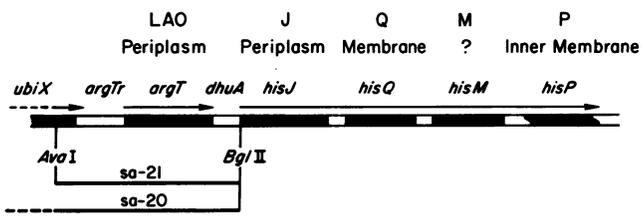

FIG. 1. Physical map of the histidine transport region of *S. typhimurium*. Each structural gene on the chromosome is represented by a solid black bar. The ends of genes are delineated by vertical borders when the precise end points are known from DNA sequence studies; wavy borders indicate that the exact end points are not yet known. Restriction endonuclease sites are marked by appropriately labeled vertical lines extending below the chromosome; the horizontal lines below the chromosome indicate specific restriction fragments subcloned. Fragment sa-20 extends to an *Eco*RI site 2.6 kilobase pairs to the left of the indicated *Ava* I site. The direction of transcription is indicated by arrows above the genes. The map is drawn to scale, *hisJ* and *argT* each being 780 nucleotides long.

Expression of both the *argT* gene and the histidine transport operon is under "nitrogen control": thus, cells respond to nitrogen limitation by increasing expression of these transport genes (9). Glutamine synthetase and several other proteins involved in the utilization and transport of "nitrogen-rich" amino acids are also under nitrogen control (refs. 9 and 11, reviewed in ref. 12) and appear to be positively regulated by the same mechanism as the histidine transport genes. This mechanism is similar in the several enteric bacteria that have been studied and is believed to operate at the level of transcription (13). A study of the regulatory regions 5′ to both the *argT* gene and the histidine transport operon is therefore important for several reasons: (*i*) to determine whether or not expression of the histidine transport operon is regulated by a mechanism similar to the transcription-attenuation mechanism of the histidine and other amino acid biosynthetic operons; (*ii*) to identify any alternative regulatory mechanism(s) involved (no nucleotide sequences from the regulatory regions for any transport operons have been published previously); and (*iii*) to identify the nucleotide sequences involved in nitrogen control, an important step in attempting to understand the mechanisms of nitrogen control at a molecular level.

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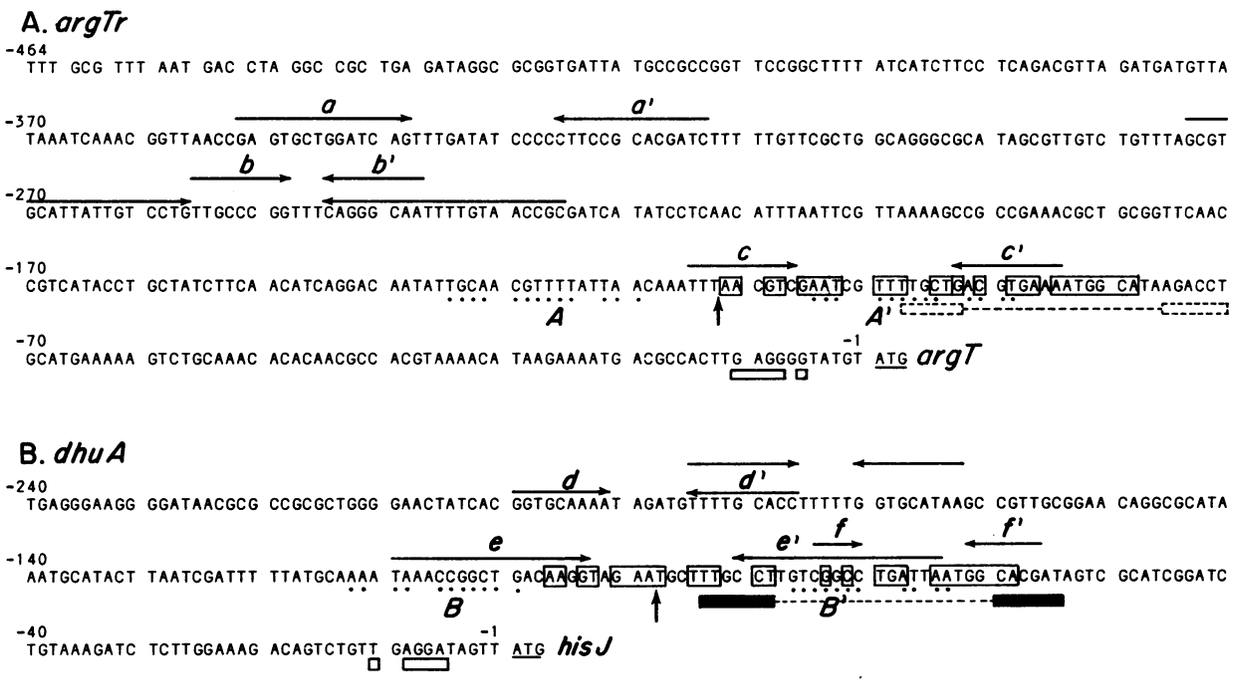

FIG. 2. Nucleotide sequences of *argTr* and *dhuA*. The sequences of both DNA strands were determined in their entirety. The antisense strand is shown (here and in all figures) with the 5′ end to the left. Symbols are used as follows: arrows, dyad symmetries forming stem and loop structures, in which $\Delta G \leq -7$ kcal/mol (1 kcal = 4.18 kJ); dots, mirror symmetries; vertical arrows, centers of mirror symmetries; open bars, ribosome-binding sites; boxed nucleotides, homologies between the two sequences. Promoters are indicated by bars joined by a broken line. The bars of the *argTr* promoter have not been filled in because its assignment is purely hypothetical (see text). Additional possible promoters were identified by their homology with the consensus promoter sequence. Those with the best homology are: in *argTr*, −136 to −108, −123 to −94; in *dhuA*, −158 to −131, −118 to −88, −93 to −63. Lower-case letters refer to the two arms of a dyad symmetry. Capital letters refer to the two arms of a mirror symmetry. Sequences can be identified that encode the following peptides (the numbers in parentheses refer to the initial nucleotide and the last nucleotide of the translation termination codon for each peptide). In *argTr*: (−421, −380) Met-Pro-Pro-Val-Pro-Ala-Phe-Ile-Ile-Phe-Leu-Arg-Arg; (−379, −368) Met-Met-Leu; (−84, −76) Met-Ala. In *dhuA*: (−188, −108) Met-Phe-Cys-Thr-Phe-Leu-Val-His-Lys-Pro-Leu-Arg-Asn-Arg-Arg-Ile-Asn-Ala-Tyr-Leu-Ile-Asp-Phe-Tyr-Ala-Lys; (−139, −128) Met-His-Thr; (−118, −68) Met-Gln-Asn-Lys-Pro-Ala-Asp-Lys-Val-Gln-Cys-Phe-Ala-Leu-Ser-Ala; (−89, −36) Met-Leu-Cys-Leu-Val-Gly-Leu-Ile-Asn-Gly-Thr-Ile-Val-Ala-Ser-Asp-Leu; (−64, −53) Met-Ala-Arg. (*A*) The *argT* structural gene begins with the ATG codon at base pair +1. The umber codon at −440 is the translation termination codon for *ubiX*, the structural gene immediately upstream from *argT*. We have obtained, but do not present, the nucleotide sequence back to −830. (*B*) The *hisJ* gene begins with the ATG codon at base pair +1. The umber codon at −240 is the translation termination codon for *argT*. The mutation *dhuA1* causes a C-to-T change at −60.

MATERIALS AND METHODS

Restriction endonucleases were purchased from Bethesda Research Laboratories or New England BioLabs and digestions were carried out as recommended by the suppliers. Polynucleotide kinase was purchased from P-L Biochemicals. [γ-^{32}P]ATP was synthesized and kindly provided by R. Myers. Plasmids pFA8 and pFA9 are derivatives of pBR322 carrying, respectively, DNA fragments sa-20 and sa-21, derived from a clone of the entire histidine transport operon (Fig. 1; ref. 14). The plasmids were constructed by standard procedures and used as a source of DNA for sequence analysis. The *dhuA1* mutation was cloned from the *S. typhimurium* chromosome into the phage vector λgt8 (15). All DNA sequence determinations were carried out by the procedures of Maxam and Gilbert (16), the sequences of both strands being determined in their entirety.

RESULTS

argTr. The nucleotide sequence of 830 base pairs 5′ to the *argT* gene has been determined. In order to locate the structural gene immediately upstream from *argT*, this sequence was translated in all six possible reading frames. Only one reading frame long enough to encode a protein (or portion thereof) was identified, stretching from the beginning of the determined sequence (base −830), to a stop codon at base −440. This sequence presumably encodes the COOH terminus of the protein immediately upstream from *argT* and is transcribed from the same strand as are both *argT* and the histidine transport operon. This gene is almost certainly *ubiX* (Fig. 1), which is known to be located no more than 900 bases upstream from the initiation codon of *argT* (15, 17). The nucleotide sequence between the COOH terminus of the putative *ubiX* gene and the NH_2 terminus of the *argT* structural gene is presented in Fig. 2*A*. A number of interesting features are apparent in this region. (*i*) Several regions showing dyad symmetry* are found which, if transcribed, could form stable hairpin loops [$\Delta G \leq -7$ kcal/mol (18)]. Dyad symmetries *a–a′* (between bases −352 and −314) and *b–b′* (between bases −256 and −238) are typical of prokaryotic transcription terminators: a stable hairpin loop followed by a run of Ts [Us in the message (19)]. Presumably one or both function in the termination of transcription of the putative *ubiX* gene. We therefore assume that the region likely to be important in regulating *argT* expression is 3′ to the latter terminator and is therefore restricted to the 236 bases between this terminator and the initiation codon for *argT*; we refer to this region as *argTr*. (*ii*) Within *argTr* there are several potential

* Hyphenated dyad symmetries (e.g., GACTNNNAGTC) will be referred to throughout as "dyad symmetries." We will refer to regions of hyphenated alphabetic symmetries (e.g., GACTNNNTCAG) as "mirror symmetries," although it must be realized that such sequences are not true symmetries due to the polarity of the DNA backbone: the bases comprising the most extended symmetrical region itself will be referred to as the "mirror proper."

Table 1. Nitrogen regulation at *dhuA*

Strain	Relevant genotype	J protein, pmol histidine bound per mg protein
TA3628	*hisΔ(ubiX argTr argT dhuA hisJ) 6704*/MA4	31
TA3623	*hisΔ(ubiX argTr argT dhuA hisJ) 6704 ntrB139*/MA4	63
TA3627	*hisΔ(ubiX argTr argT dhuA hisJ) 8907*/MA4	40
TA3624	*hisΔ(ubiX argTr argT dhuA hisJ) 8907 ntrB139*/MA4	72

MA4 is a recombinant derivative of M13*mp2* carrying the histidine transport genes, but not *argT* (see text). Histidine-binding activity (at 10 nM [^{3}H]histidine) was assayed in shock fluids by equilibrium dialysis as described (5). The increase in binding activity due to the *ntrB139* mutation is slightly less in strains carrying *hisJ* on the phage than that obtained with *hisJ* on the chromosome (9). This is probably due to a slight inhibitory effect of the M13 phage on cell growth.

promoters showing homology with the consensus sequence for prokaryotic promoters (19); the promoter indicated in Fig. 2*A* is a possible candidate for being the *in vivo* promoter by analogy with the location of the *dhuA* promoter (see below). (*iii*) A region of mirror symmetry,* *A–A′* (centered on bases −112/−113) is also indicated and may be important in the regulation of *argT* expression (see *Discussion*).

dhuA. The regulatory region for the histidine transport operon, *dhuA*, is located between the *argT* and *hisJ* structural genes (Fig. 1; ref. 7). The entire nucleotide sequence of this region, consisting of only 240 base pairs, is presented in Figure 2*B*. It has been shown elsewhere that the coding sequences delimiting this region are indeed the structural genes *argT* and *hisJ* (10). A number of interesting features are also apparent in this regulatory sequence. (*i*) Several regions showing dyad symmetry are found. The dyad symmetry *d–d′* (between bases −200 and −177) is typical of transcription terminators and presumably is the transcription terminator for the *argT* gene. (*ii*) Several potential promoters can be identified. However, the sequence of the regulatory (promoter-up) mutation *dhuA1*, which results in a 10-fold increase in the synthesis of the histidine transport proteins (20), has been determined: this mutation causes a C-to-T change at base −60, altering the Pribnow box of one of the potential promoters to a sequence more similar to that of the consensus promoter sequence. This indicates that this promoter is the *in vivo* promoter, although the possibility remains that the *dhuA1* mutation may simply have created a new promoter sequence. It should also be mentioned that the C-to-T change in *dhuA1* totally destabilizes the hairpin loop that could potentially be formed by the dyad symmetry *f–f′* (between bases −75 and −57), increasing the calculated ΔG value from −11.0 to −1.9 kcal/mol. This might result in increased transcription, and therefore increased synthesis of the transport proteins, by disruption of an as yet unknown regulatory mechanism. (*iii*) A region of mirror symmetry, *B–B′*, is found, centered on bases −88/−89 (see *Discussion*).

In both the *dhuA* and *argTr* there are a number of potential coding sequences for small peptides, listed in the legend to Figure 2. Typical ribosome-binding sites (21) can be identified immediately preceding both *argT* and *hisJ*.

Nitrogen Regulation. It is important to demonstrate that *argT* and the histidine transport operon are independently regulated by nitrogen availability. As *argT* is only 240 bases upstream from the histidine transport operon, and the genes are transcribed in the same direction, it is possible that regulation is mediated only at the *argTr* locus. Thus, the effect of a nitrogen regulatory mutation, *ntrB139* [previously called *glnR139* (9, 13)], on the level of J protein was studied in the absence of *argTr*. Pairs of strains, differing only in the presence or absence of mutation *ntrB139*, were constructed, each with a chromosomal deletion eliminating *ubiX*, *argTr*, *argT*, *dhuA*, and *hisJ*. The entire histidine transport operon was introduced into each strain as a fragment of *S. typhimurium* DNA inserted into the phage M13*mp2* (22). This recombinant phage, MA4 (details of phage construction to be published elsewhere), does not carry *argTr*. The level of J protein in each of these strains was determined. Table 1 shows that in these *argTr*$^-$ strains the concentration of the J protein is increased by the *ntrB* mutation, demonstrating that nitrogen regulation is mediated at *dhuA* independently of *argTr*.

DISCUSSION

In order to better understand the mechanisms involved in regulating expression of the amino acid transport operons, we have determined the nucleotide sequences of the regulatory regions of two such operons, *argTr*, which regulates the *argT* gene, and *dhuA*, which regulates the histidine transport operon. Because amino acid transport systems serve a similar function to the amino acid biosynthetic operons in supplying the cell with building blocks for protein synthesis, it seems plausible that similar mechanisms might be involved in regulating expression of the transport and the biosynthetic operons. It is well known that several amino acid biosynthetic operons, including that for histidine, are regulated by a transcription attenuation mechanism (1–3). However, it is apparent from our data (Fig. 2) that neither *argTr* nor *dhuA* contains any of the features typical of the regulatory regions of the amino acid biosynthetic operons. (*i*) Neither region has a typical transcription termination sequence (apart from those responsible for termination of the preceding structural genes) appropriately located with respect to a series of overlapping dyad symmetries. (*ii*) Although several sequences that could potentially encode leader peptides can be identified, none of these sequences seems likely to be of importance in regulation because: (*a*) the starts of all of the potential peptides are located 5′ to the probable transcription start sites and could, therefore, not be transcribed or translated; (*b*) none of these peptides contain multiple tandem codons for amino acid(s) related to the operon. In addition, there is no similarity in the location or composition of the peptides between *argTr* and *dhuA*. Thus, the absence from *argTr* and *dhuA* of any of the typical features of the regulatory regions for the amino acid biosynthetic operons implies that attenuation, mediated by the premature termination of transcription, is not the mechanism by which expression of these genes is regulated. This is in agreement with the observation that regulatory mutations (*hisR*, *hisS*, and *hisT*) known to increase the expression of the histidine biosynthetic operon (23) either have no major effect on the expression of the histidine transport genes or actually reduce histidine transport slightly (unpublished observation).

Having excluded a classical attenuation mechanism of regulation, we compared *argTr* and *dhuA* with each other in order to identify features common to the two regions that might suggest an alternative regulatory mechanism and, in particular, to

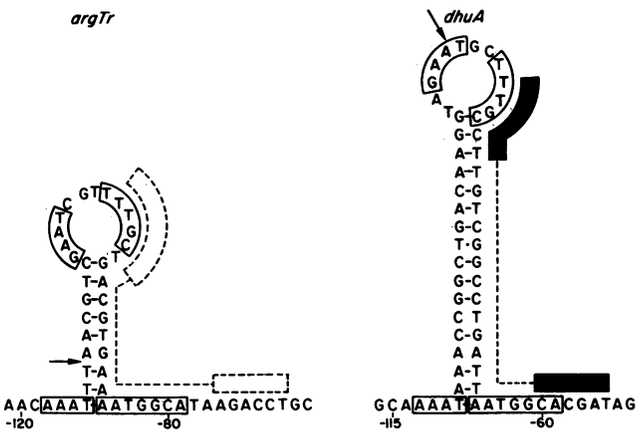

FIG. 3. Possible stem–loop structures in *argTr* and *dhuA*. Base numbering and symbols are as in Fig. 2. The boxes indicate nucleotides identical in the two stem–loop structures (see text). Arrows indicate the centers of the mirror symmetries.

identify which nucleotide sequences are specifically involved in regulating these genes in response to nitrogen (9). This comparison revealed three interesting features common to both regions, all of which are located in the proximity of the presumed promoters and are therefore appropriately placed such that they could serve to regulate the transcription, the translation, or both, of these genes. (*i*) A dyad symmetry with some unusual characteristics is found in both *argTr* (*c–c'*) and *dhuA* (*e–e'*). (*ii*) Mirror symmetries occur in *argTr* (*A–A'*) and in *dhuA* (*B–B'*) (dotted bases in Fig. 2). (*iii*) A region of homology between *argTr* and *dhuA* is found in which 25 bases out of 39 (64%) are identical (these are boxed in Fig. 2). These three features are discussed below.

Stem and Loop Structures. The sequences of the dyad symmetries *c–c'* and *e–e'* in *argTr* and *dhuA*, respectively, are particularly interesting because they can potentially form similar stem and loop structures (illustrated in Fig. 3). These structures show considerable homology: particularly striking are the 11 identical bases brought together at the "foot" of each stem and the similarity in base composition of the loop. The sequence A-A-A-T-A-A-T-G-G-C-A is formed at the foot of the stems by bringing together sequences otherwise separated by considerable distances (29 bases in *argTr* and 44 bases in *dhuA*). We have searched sequences from other operons for related structures. Interestingly, the regulatory region of the histidine biosynthetic operon† contains a stem and loop structure forming an almost identical foot and also containing the sequence T-T-G-C in its loop. The relationship of this stem and loop structure (at base pairs −44 to −30) with respect to the promoter in the histidine biosynthetic operon is also similar to the relationship found in *dhuA*. The existence of this structure in three regulatory regions strongly indicates that it serves a specific function, such as being the recognition site for a common regulatory signal, although as yet it is premature to propose a specific function. It should be pointed out that presumably these stem and loop structures are not formed in the messenger RNA because they are located upstream relative to the promoters. However, it is known that stem and loop structures can form spontaneously in negatively supercoiled DNA (24, 25); alternatively, such structures might be formed or stabilized as the result of a protein interacting with specific sequences (e.g., the "foot").

Mirror Symmetry. The identification of a mirror symmetry in the proximity of the promoter in both *argTr* and *dhuA* is also particularly interesting. We have also found mirror symmetries in the neighborhood of the promoters of the histidine† and tryptophan (26) biosynthetic operons and of the *lacI* gene (27), and in the regulatory region of *glnA*, the structural gene for glutamine synthetase (the promoter for which has not yet been identified).‡ The fact that such mirror symmetries have been found in the promoter regions of several operons indicates that they may serve important functions, such as being the recognition sites for specific regulatory proteins (discussed below). The possibility of an interaction between a protein and a region of mirror symmetry is a concept that has not been proposed previously, to our knowledge. A mirror symmetry does not allow secondary structures to form in either DNA or RNA; therefore the primary nucleotide sequence must be important for specific recognition. The existence of a symmetry in the base sequence implies that specific recognition involves a dimeric (or multimeric) protein, as is seen for protein recognition sites that display dyad symmetry [e.g., the *lac* repressor (28) or the *cro* protein from bacteriophage λ (29)]. However, mirror symmetry is not a true symmetry because the polarity of the sugar-phosphate backbone of the two repeats is different. Thus any protein–DNA interaction involving a mirror symmetry must be independent of the polarity of the backbone. If indeed such an interaction between a dimeric protein and a mirror symmetry is possible in double-stranded DNA, this interaction must be very different from known protein–DNA interactions involving dyad symmetries. Preliminary model-building and cylindrical projections§ of the distribution of functional groups (i.e., hydrogen bond donors and acceptors) indicate that, at least for those mirror symmetries analyzed, the distribution of functional groups does show twofold symmetry in the minor, though not in the major, groove. These mirror sequences could therefore serve as a symmetrical recognition site for a dimeric protein. As an alternative to interacting with the DNA double helix, such an interaction might involve single-stranded DNA. In this regard, it is interesting to note that the mirror symmetries are found in promoter regions, where the DNA strands are presumably separated during transcription.

Homology. The third feature of interest is the region of homology between *argTr* and *dhuA* shown by the boxed-in bases in Fig. 2. The structural genes *argT* and *hisJ* are 70% homologous and are believed to have arisen from a common ancestral gene as a result of gene duplication (10). Thus, the noncoding regions 5′ to these two genes might also be expected to show homology. However, a detailed comparison of *argTr* with *dhuA* failed to reveal any significant overall homology, indicating either that the duplication event giving rise to these two genes occurred sufficiently long ago to allow considerable changes to accumulate in the noncoding regions surrounding the genes or that the duplication did not include this regulatory region. The former view is supported by calculations showing that the large number of silent codon differences (those not effecting an amino acid change) between the *argT* and *hisJ* structural genes is equivalent to a randomization of those bases that are under little or no selective pressure (10). Thus, the specific region of homology observed between the *argT* and *hisJ* regulatory regions probably reflects a common function.

Nitrogen Regulation. Because *argT* and the histidine transport operon are under nitrogen control, the question arises as to which features in their regulatory regions are important for nitrogen regulation. The gene encoding glutamine synthetase, *glnA*, is also under nitrogen control but is otherwise completely

† This sequence was kindly made available to us by W. Barnes.

‡ The nucleotide sequence 5′ to *glnA* was kindly made available to us prior to publication by Jean Brenchley.

§ Kindly performed by S.-H. Kim.

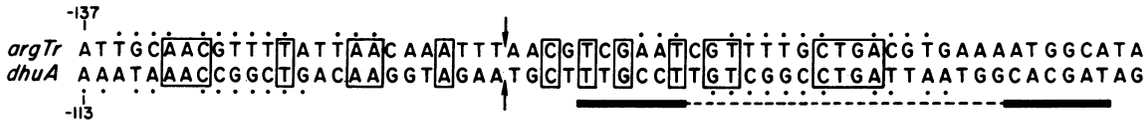

FIG. 4. Comparison of mirror sequences in *argTr* and *dhuA*. Base numbering and symbols are the same as in Fig. 2.

unrelated to *argT* and the histidine transport operon. Thus, any feature common to the regulatory region for *glnA*, as well as to *argTr* and *dhuA*, may be important for nitrogen regulation. We consider it unlikely that the "stem–loop–foot" structure discussed above is involved: no such structure can be found in the regulatory region for *glnA*, and although a similar structure is found in the histidine biosynthetic operon, this operon is not under nitrogen control [as determined by assays of histidinol-phosphate phosphatase, either in strains carrying the nitrogen regulatory mutation *ntrB139* or in the wild type grown on a poor nitrogen source (data not presented)]. Rather, we speculate that the mirror symmetries identified in *argTr* and *dhuA*, as well as the *glnA* regulatory region, are involved in the mechanism of nitrogen regulation. In support of this view is the fact that the mirror symmetry in *dhuA* shares extensive homology with the mirror symmetry in the *glnA* regulatory region (10 out of 14 bases in the mirror proper* are identical). Although the homology between the mirror symmetries in *argTr* and *dhuA* is poorer (5 out of 14 in the mirror proper, as shown in Fig. 4), if functional groups rather than the specific bases are considered, the similarity may be considerably better than appears at first sight. It should also be pointed out that there is some overall homology between *argTr* and *dhuA* in the general region of the mirrors (bases boxed in Fig. 4). Although no other nucleotide sequences of regulatory regions responding to nitrogen control have been published, we have not found mirror symmetries sharing homologies with ours in a screen [by computer (30) and manually] of the regulatory regions of more than 30 genes that are not under nitrogen control.

Kustu and her coworkers have proposed that nitrogen control may operate in an analogous manner to the cAMP-binding protein/cAMP system for carbon regulation (13). In *S. typhimurium*, three regulatory genes have been identified that play a pleiotropic role in nitrogen control, *ntrA* (formerly *glnF*) and *ntrB* and *ntrC* [formerly considered as a single gene, *glnR* (13)]. Under conditions of nitrogen limitation, it is proposed that either the *ntrA* gene product itself, or a low molecular weight coregulator produced by it, promotes the interaction of the *ntrB/ntrC* gene products with specific nitrogen regulatory regions on the chromosome, leading to an increase in transcription. It is possible that the mirror sequences in nitrogen-regulated regions are the recognition sites for such an interaction. As discussed above, preliminary model building with these mirror symmetries shows that symmetrical protein–DNA interactions are possible at these sites.

Clearly, the functional significance of the mirror symmetries and of any of the other features discussed remains to be resolved. A better understanding of the mechanism of nitrogen control and of the regulation of expression of these transport genes will depend upon the isolation of appropriate mutants, upon DNA–protein binding studies, *in vitro* transcription and translation, and comparison with other similarly regulated genes.

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