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The *nadI* Region of *Salmonella typhimurium* Encodes a Bifunctional Regulatory Protein

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Mutants of the *nadI* and *pnuA* genes were independently isolated on the basis of defects in repression of NAD biosynthetic genes and defects in transport nicotinamide mononucleotide (NMN). The mutations map at min 99 on the *Salmonella* chromosome, and the affected regions appear to be cotranscribed. Some pairs of *nadI* and *pnuA* mutations complement, suggesting the existence of independent functions. However, *cis/trans* tests with particular mutations provide evidence that both repressor and transport functions are actually performed by a single bifunctional protein. (This result confirms sequencing data of Foster and coworkers [J. W. Foster, Y. K. Park, T. Fenger, and M. P. Spector, J. Bacteriol. 172:4187–4196, 1990]). We have designated the gene for this bifunctional protein *nadI* and distinguish the regulatory and transport defects with phenotypic designations (R and T). When a *nadI*(R^- T^+) mutation (eliminating only repression function) is placed *cis* to a superrepressor mutation, *nadI*(R^s T^-), the superrepression phenotype is lost. In contrast, placement of R^- and R^s T^- mutations in *trans* allows full superrepression. This result suggests that the transport function (eliminated by the R^s T^- mutation) and the repression function are provided by the same protein. Insertion mutations in the promoter-proximal repressor region of the *nadI* gene eliminate transport function unless the inserted element can provide both for both transcription and translation start signals; this finding suggests that there is no transcriptional or translational start between the regions encoding repression and transport functions.

In *Salmonella typhimurium*, NAD is synthesized both by a de novo pathway and by two salvage pathways. These pathways are diagrammed in Fig. 1 of the accompanying report (34). Transcriptional regulation of this pathway is accomplished by a repressor, encoded by the *nadI* gene; this repressor controls transcription of genes for the first two biosynthetic enzymes, *nadB* and *nadA* (10, 14, 17, 32). The *nadI* repressor gene maps at min 99 of the *Salmonella* chromosome, very near a gene involved in nicotinamide mononucleotide (NMN) transport, *pnuA* (10, 14, 15, 17, 20, 29, 32). Previous work on the functional and genetic relationship between the *nadI* and *pnuA* genes led to the conclusion that the region contains either a single bifunctional protein or perhaps two independent genes in one operon (10, 14, 32). Foster et al. (who designate this region *nadR*) have found that the DNA sequence for this region includes a single open reading frame (15a). We present here genetic data supporting their conclusion, providing in vivo evidence for the existence of a single bifunctional gene and further characterizing the genetic structure of this locus.

We have designated the gene for the inferred bifunctional protein *nadI* and distinguish the regulatory and transport defects with phenotypic designations (R and T). In the accompanying paper (34), we present evidence that both functions of this protein are regulatory. One function (R) controls transcription of two biosynthetic genes; the other (T) serves to regulate the activity of the NMN transport function. Both functions appear to exert their control in response to internal NAD (or NADP) levels.

MATERIALS AND METHODS

Bacterial strains. All strains used in this study are derived from *S. typhimurium* LT2 and are listed in Table 1. Mu dA refers to a conditionally transposition-defective derivative (18) of the original Mu d1(Lac Ap^r) phage of Casadaban and Cohen which forms operon fusions (4). Mu dJ refers to a transposition-defective mini-Mu phage, Mu d1-1734(Lac Km^r), constructed by Castilho et al. (5); this phage lacks transposition functions and carries kanamycin resistance. Tn*10d*(Tc) refers to a small transposition-defective derivative of Tn*10* (Tn*10* Del16 Del17 Tet^r) constructed by Way et al. (31). Tn*10d*(Cm) refers to a transposition-defective derivative of transposon Tn*10* constructed by Elliott and Roth (13).

Media. The E medium of Vogel and Bonner (30), supplemented with 0.2% glucose, was used as the minimal medium. Difco nutrient broth (NB; 8 g/liter with 5 g of NaCl per liter) was used as the rich medium. Difco agar was added at a final concentration of 1.5% for solid medium. Nutrients to feed auxotrophs were included in minimal media at final concentrations described by Davis et al. (11); exceptions are indicated in the text. Antibiotics were added to media at the following final concentrations: ampicillin (sodium salt), 30 μg/ml in NB and 15 μg/ml in E medium; tetracycline hydrochloride and chloramphenicol, 20 μg/ml in NB and 10 μg/ml in E medium; and kanamycin sulfate, 50 μg/ml in NB and 125 μg/ml in E medium. All antibiotics were obtained from Sigma Chemical Co.. Media containing ampicillin were prepared fresh before use. The chromogenic β-galactosidase substrate 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-Gal) was dissolved in *N*,*N*-dimethyl formamide (20 mg/ml) and added to media at a final concentration of 25 μg/ml. Similarly, the chromogenic substrate for alkaline phosphate, 5-bromo-4-chloro-3-indolylphosphate, toluidine salt (X-P), was dissolved in *N*,*N*-dimethyl formamide (20 mg/ml) and added to media to a final concentration of 50 μg/ml.

Transductional methods. The high-frequency, generalized transducing bacteriophage P22 mutant HT105/1 *int-201* was

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TABLE 1. Strains

Strain[a]	Genotype[b]
TT8370	*thr-458*::Mu dA
TT8371	*thr-469*::Mu dA
TT10091	*nadB227*::Mu dA *nadI260*(R^- T^+)
TT11420	*nadI*^{s}*509 srl-202*::Tn*10recA1*
TT12933	*nadB499*::Mu dJ *pncA278*::Tn*10d*(Cm) *DEL787C(serB-pnuA)*
TT12940	*nadB499*::Mu dJ *pncA278*::Tn*10d*(Cm) *DEL794(serB-pnuA)*
TT12946	*nadB499*::Mu dJ *pncA278*::Tn*10d*(Cm) *DEL800(serB-nadI)*
TT12952	*nadB499*::Mu dJ *pncA278*::Tn*10d*(Cm) *DEL806(serB-nadI)*
TT12953	*nadB499*::Mu dJ *pncA278*::Tn*10d*(Cm) *DEL807(serB-nadI)*
TT12958	*nadB499*::Mu dJ *pncA278*::Tn*10d*(Cm) *DEL812(serB-nadI)*
TT12963	*nadB499*::Mu dJ *pncA278*::Tn*10d*(Cm) *DEL817(serB-nadI)*
TT12967	*nadB499*::Mu dJ *pncA278*::Tn*10d*(Cm) *DEL821(serB-nadI)*
TT12971	*nadB499*::Mu dJ *pncA278*::Tn*10d*(Cm) *DEL825(serB-nadI)*
TT13120	*nadB215*::Tn*10 pncA278*::Tn*10d*(Cm)
TT13278	*nadI*^{s}*509pncA278*::Tn*10d*(Cm)
TT13516	*pncA278*::Tn*10d*(Cm) *nadI556*::Mu dK *nadI*^{s}*509*
TT15490	*nadB215*::Tn*10 pncA278*::Tn*10d*(Cm) *serB9*
TT15491	*nadB215*::Tn*10 pncA278*::Tn*10d*(Cm) *nadI559*::Mu dJ(R^- T^-)
TT15493	*nadB215*::Tn*10 pncA278*::Tn*10d*(Cm) *nadI561*::Mu dJ(R^- T^+)
TT15922	*nadA542 pncA278*::Tn*10d*(Cm) *nadI563*::Mu dJ(R^- T^-)
TT15924	*nadA542 pncA278*::Tn*10d*(Cm) *nadI561*::Mu dJ(class II)
TT15926	*nadA542 pncA278*::Tn*10d*(Cm) *nadI562*::Mu dJ(class I)
TT15998	*nadB227*::Mu dA *pncA278*::Tn*10d*(Cm) *DUP728*[(*serB9 nadI260*(R^- T^+))*Tn*10**(nadI*^{s}*511*(R^s T^-))]
TT16005	*nadB227*::Mu dA *pncA278*::Tn*10d*(Cm) *DUP728*[(*serB9 nadI260*(R^- T^+))*Tn*10**(nadI299*(R^+ T^-))]
TT16032	*nadB499*::Mu dJ *pncA278*::Tn*10d*(Cm) *serB1463*::Tn*10 nadI*^{s}*511*
TT16039	*nadB227*::Mu dA *pncA278*::Tn*10d*(Cm) *DUP728*[(*serB9*)*Tn*10**(nadI260*(R^- T^+) *nadI*^{s}*511*(R^s T^-))]
TT16156	*nadA542 pncA278*::Tn*10d*(Cm) *DEL1100(nadI561–thr-469)*::Mu dB
TT16176	*nadA379*::Tn*10d*(Tc) *pncA278*::Tn*10d*(Cm) *DEL1620(nadI553–thr-469)*::Mu dB
TT16201	*nadB227*::Mu dA *pncA278*::Tn*10d*(Cm)
TT16202	*nadB227*::Mu dA *pncA278*::Tn*10d*(Cm) *nadI*^{s}*509*
TT16205	*nadB227*::Mu dA *pncA278*::Tn*10d*(Cm) *nadI553*::Mu dK(type II)
TT16206	*nadB227*::Mu dA *pncA278*::Tn*10d*(Cm) *nadI553*::Mu dK(type II) *nadI*^{s}*509*
TT16207	*nadB227*::Mu dA *pncA278*::Tn*10d*(Cm) *nadI554*::Mu dK (type I)
TT16208	*nadB227*::Mu dA *pncA278*::Tn*10d*(Cm) *nadI554*::Mu dK(type I) *nadI*^{s}*509*
TT16211	*nadA542 pncA278*::Tn*10d*(Cm) *DEL1625(serB-nadI560)*::Km^r
TT16214	*nadA542 pncA278*::Tn*10d*(Cm) *DEL1628(serB-nadI561)*::Km^r
TT16217	*nadA542 pncA278*::Tn*10d*(Cm) *serB1466*::Tn*10d*(Tc) *nadI560*::Mu dJ(R^- T^-)
TT16218	*nadA542 pncA278*::Tn*10d*(Cm) *serB1466*::Tn*10d*(Tc) *nadI563*::Mu dJ(R^- T^-)
TT16219	*nadA542 pncA278::Tn10d*(Cm) *serB1466*::Tn*10d*(Tc) *nadI561*::Mu dJ(type II)
TT16220	*nadA542 pncA278*::Tn*10d*(Cm) *serB1466*::Tn*10d*(Tc) *nadI562*::Mu dJ(type I)
TT16222	*nadA542 pncA278*::Tn*10d*(Cm) *serB1463*::Tn*10 nadI563*::Mu dJ(R^- T^-)
TT16223	*nadA542 pncA278*::Tn*10d*(Cm) *serB1463*::Tn*10 nadI561*::Mu dJ(type II)
TT16224	*nadA542 pncA278*::Tn*10d*(Cm) *serB1463*::Tn*10 nadI562*::Mu dJ(type I)
TT16234	*nadB227*::Mu dA *pncA278*::Tn*10d*(Cm) *nadI260*(R^- T^+)

[a] All strains are derivatives of *S. typhimurium* LT2 and were either constructed in the course of this work or obtained from the *Salmonella* strain collection, University of Utah.

[b] Nomenclature is as described by Demerec et al. (12), Chumley et al. (7), and Schmid and Roth (26).

used for all transductional crosses. This phage was derived by Roberts (25a) from the P22 HT105/1 phage of Schmieger (27). To select for the inheritance of the Km^r marker of Mu dJ and the Cm^r marker of Tn*10d*(Cm), the transduction mixture of cells and phages was spread on NB plates and incubated overnight before replica printing to selective plates. In all other crosses, selective plates were spread directly with 2×10^8 cells and 10^8 to 10^9 phages. Phage-free transductant clones were identified by nonselective streaking on green indicator plates (6). Phage-free clones form light-colored colonies on green indicator plates; phage sensitivity was confirmed by cross-streaking with P22 H5 phage, a clear-plaque mutant of P22.

Mutagenesis. Insertion mutations generated by Mu dJ and Mu dK were isolated by the *cis* complementation method described previously (19).

Fine-structure deletion mapping. Deletion mapping was done by phage P22-mediated transductional crosses. A fresh overnight culture of a strain carrying deletion from the *serB* to *nadI* region (10^9 cells per ml) was washed, concentrated 10-fold by centrifugation, and then infected at a multiplicity of 10 with transducing lysates grown on *serB*$^+$ *nadI*(T^-) mutant strains. Before mapping, *nadI*(R^- T^+) insertion mutants were converted to *nadI*(R^- T^-) deletions (see text). Transductants that are Ser^+ and able to transport NMN were selected on minimal medium with 10^{-4} M NMN. After 2 days of incubation on selective medium, a set of recombinant colonies was patched and all markers were scored by replica printing. A wild-type donor typically yields more than 10^5 recombinant colonies ($SerB^+$ NMN^+) per plate under these conditions. A cross was scored as negative if no recombinants were seen on any of five plates. Thus, the resolution of the map is 2×10^{-6}.

Enzyme assay. β-Galactosidase activity was determined as described by Miller (23), with sodium dodecyl sulfate-chloroform-permeabilized cells. The β-galactosidase activity is

reported as nanomoles per minute per optical density unit (at 650 nm) of cells.

Construction of *nadI*(R^- T^+) and *nadI*(R^s T^-) double mutants. The double mutant used in the *cis/trans* test was constructed by transducing a *serB nadI511*(R^s T^-) strain (TT16032) with phage grown on the *nadI260*(R^- T^+) mutant (TT10091); Ser^+ transductants were scored for inability to use NMN (*nadI511*; R^s T^-) and constitutive expression of the *nadB*::*lac* fusion (inheritance of *nadI260*). Recombinants with both phenotypes were expected to be *nadI260 nadI*^{s}*511* double mutants; this structure was confirmed by crosses which recovered each of the individual mutations from the double mutant.

RESULTS

Isolation of *nadI* insertion mutations that form *lac* operon and protein fusions. Insertions of elements Mu dJ and Mu dK in the *nadI* gene were identified by a defect in repressor function. These insertions were isolated as prototrophic revertants of *nadI509*(R^s T^-) mutant strains (TT13278 and TT11420). The parental *nadI509*(R^s T^-) mutation causes a Nad^- auxotrophic phenotype by tight repression of NAD synthetic genes (32). Revertant mutations permitting pyridine-independent growth map at the *nadI* locus and have lost repressor function. These mutants were screened for their ability to transport NMN. Since the parent *nadI*(R^s) mutation itself causes a transport-defective phenotype, the new *nadI*(R^-) insertions had to be genetically separated before their transport phenotype could be checked. Some, but not all, of the separated *nadI*(R^-) insertion mutations eliminated NMN transport; the mutations were designated *nadI*::Mu d(R^- T^+) (repressor deficient and transport proficient) or *nadI*::Mu d(R^- T^-) (repressor deficient and transport deficient), respectively. The *nadI*::Mu d(R^- T^+) insertions that form blue colonies on medium containing X-Gal have formed *lac* operon fusions or *lacZ* protein fusions to the *nadI* gene.

Simple transport-deficient Mu dJ insertions were isolated from a *nadI*$^+$ *nadB pncA* parent (TT13120) as derivatives that fail to use NMN as a pyridine source. All such insertions are either in the *pnuC* gene (located near the *nadA* gene at min 17) or in the *nadI* gene near *serB* at min 99. The *nadI*(T^-) insertions were all found to be deficient in the repressor function; i.e., they are all *nadI*::Mu dJ(R^- T^-) insertions. The insertion mutants that formed blue colonies on medium containing X-Gal were presumed to be transcribed by the *nadI* promoter. Expression of the *nadI*(R^- T^-) and *nadI*(R^- T^+) fusions is not influenced by exogenous nicotinic acid, nicotinamide, or NMN (see below).

Orientation of *nadI* transcription. By using a method described previously (7, 22), an F′ *lac* plasmid was inserted into the chromosome by recombination between a chromosomal *nadI*::Mu d(*lac*) element and the *lac* sequences of the plasmid. The direction of chromosome transfer by the resulting Hfr strain (data not shown), indicated that both *nadI*::Mu dJ(R^- T^+) and *nadI*::Mu dJ(R^- T^-) fusions were transcribed in a clockwise direction (*serB* → *nadI* → *thr*); that is, transcription of the *nadI* region starts at the end nearest the *serB* gene and proceeds toward the *thr* locus. The orientation of transcription was confirmed by studying spontaneous deletion mutations in the region as described below.

Map order of *nadI*(R^- T^+) and *nadI*(R^+ T^-) insertions. Repressor mutations, *nadI*(R^-), and NMN transport mutations, nadI(T^-), previously designated *pnuA*, both map between the *serB* and *thr* genes (10). However, their map order with respect to each other is uncertain. We determined their order by the phenotypes of deletion mutations and by three-factor crosses. The results (see below) support the map order (read clockwise) *serB-nadI*(R)-*nadI*(T)-*thr*.

The *nadI*::Mu dJ(R^- T^+) insertion mutants can use NMN as a pyridine source. Deletions extending from *serB* to a *nadI*::Mu dJ(R^- T^+) insertion (e.g., TT16214) remove the material between *serB* and the *nadI*::Mu dJ insertion, including the *nadI* promoter, but leave the Km^r determinant of Mu dJ and all of the *nadI* material downstream of the Mu dJ insertion site. These deletion strains still express the *nadI* transport function, since they can grow on 10^{-4} M NMN as does the parent insertion mutant.

Deletion mutants generated by recombination between the Mu dJ sequences of mutations *nadI*::Mu dJ(R^- T^+) and *thr*::Mu dA (e.g., TT16156 and TT16176) were tested for growth on 10^{-4} M NMN. These mutants have lost material between *nadI*::Mu dJ and *thr*::Mu dA, including the distal end of the *nadI* gene; they retain *nadI* material upstream from the Mu dJ insertion site. Consistent with these findings, the *nadI*::Mu dJ-*thr*::Mu dA deletion mutants have become NMN^-.

The order of functions in this region has also been tested by three-factor crosses involving a *nadI*::Mu dJ(R^- T^+) insertion mutant (TT16205) and a *nadI*(R^- T^+) point mutant (TT16234) used as recipients and a *serB*::Tn*10d*(Tc) *nadI*(R^+ T^-) strain (TT16176) used as the donor. The order of these mutations supports the order *serB-nadI*(R)-*nadI*(T)-*thr*.

The *nadI*(R^s) mutations directly affect the NMN transport function. The *nadI*(R^s) mutants were isolated because they show constitutive repression of the synthetic genes *nadA* and *nadB*; this repression is sufficient to cause an auxotrophic phenotype. All *nadI*(R^s) mutants also prove to be defective in NMN utilization (32). (In the accompanying paper [34], we show that the transport deficiency is not due to a failure to express the regulated transport function *pnuC*.) The transport defect of *nadI*(R^s) mutations suggested that mutations affecting repression in this way might directly affect the transport function of a bifunctional NadI protein.

In the course of mutant isolation (described above), it was found that *nadI*::Mu dK(R^- T^+) mutations suppress the auxotrophic phenotype of *nadI*(R^s) mutations, presumably by preventing superrepression. However, these double mutants (like the single R^s parental mutant) fail to use NMN. Thus, the *nadI*(R^s) mutation continues to shows its effect on NMN transport when in combination with a mutation that abolishes the repressor function and restores expression of *nadI* biosynthetic genes. This indicates that the *nadI*(R^s) mutations do not lose transport function because of superrepression but may directly affect the transport region of the *nadI* protein.

Three-point crosses and a deletion map of the *nadI* region (see below) clearly demonstrate that *nadI*^{s} mutations map at the promoter-distal (transport) end of the *nadI* gene. The existence of *nadI*(R^s) mutations with a transport defect supports the idea of a single NadI (repression-transport) protein but does not eliminate the possibility of a complex of separate repressor and transport proteins.

Deletion map of the *nadI-pnuA* region. A deletion map of the *nadI* region was constructed by using spontaneous deletion mutants, isolated as Tc^s revertants of a *serB*::Tn*10d*(Tc) strain (TT15490). Mutants selected as Tc^s were screened for the ability to use NMN (transport) and for constitutive expression of the *nadB499*::Mu dJ fusion (repression). All *serB-nadI* deletion mutants are (R^- T^-).

The *nadI* insertion or point mutation strains used in map construction are all *serB*$^+$. Point mutants with an NMN^-

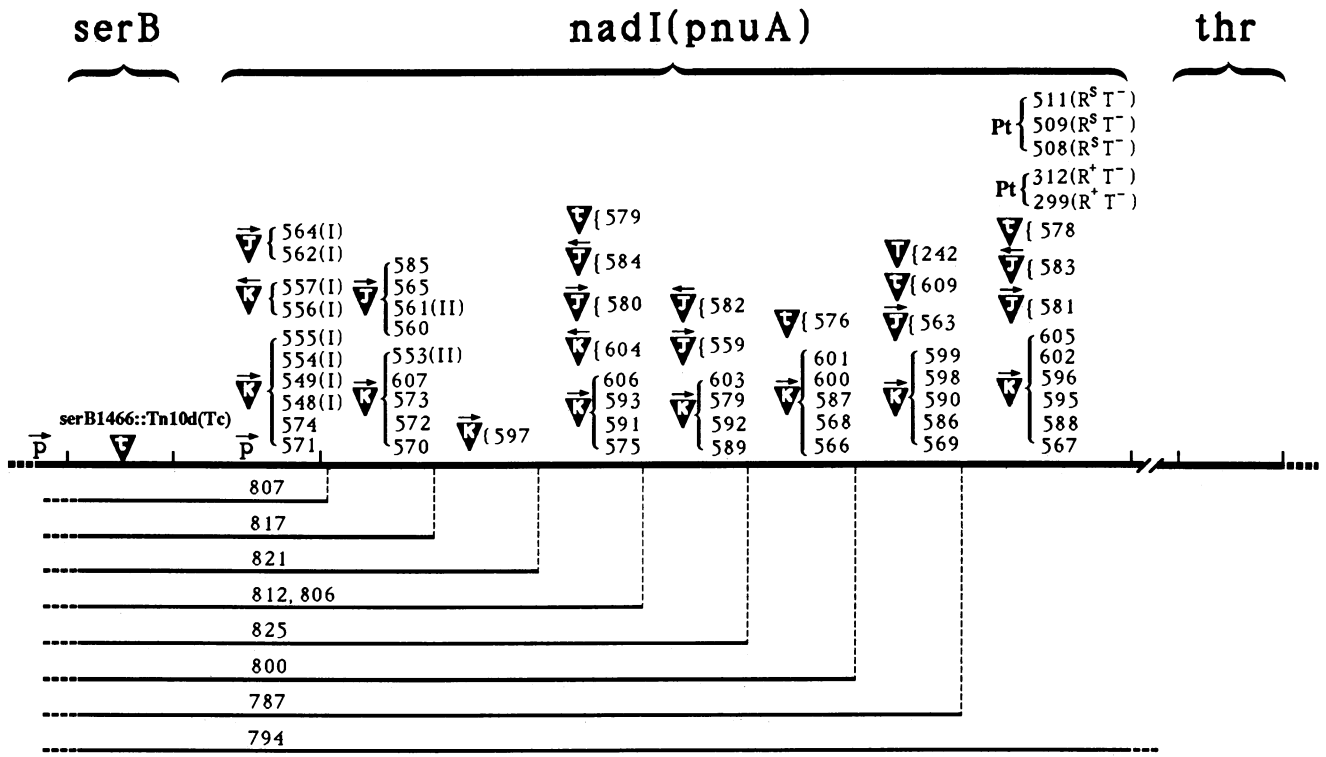

FIG. 1. Deletion map of the *nadI-pnuA* region. Deletion mutations, listed below the heavy horizontal line designating the chromosome, were all isolated as spontaneous tetracycline-sensitive revertants of the *serB1644*::Tn*10d*(Tc) mutant. The allele numbers are indicated above each deletion. The deletion endpoints are indicated by vertical dotted lines. The types of insertion and point mutations mapped are indicated as allele numbers above each deletion interval. The letter J in a dark triangle indicates a Mu dJ insertion; the letter K designates a Mu dK insertion. The arrows above these insertion symbols indicate the orientation of their *lac* operon. A dark triangle with a lowercase "t" denotes a Tn*10d*(Tc) insertion; an uppercase "T" indicates a Tn*10* insertion. Point mutations are designated **Pt**. The phenotypes of insertion and point mutations are shown in parentheses, with R indicating repression and T indicating NMN transport ($R^- T^-$, $R^- T^+$, or $R^+ T^-$). Superrepressor mutations are designated $R^s T^-$ since they all show a transport defect as well as superrepression. For Mu d insertions, type I indicates a *nadI*($R^{\pm} T^+$) insertion and type II indicates *nadI*($R^- T^+$) insertion.

phenotype (either $R^+ T^-$, $R^- T^-$, or $R^s T^-$) were directly crossed with deletion mutants, selecting for growth on NMN as a pyridine source and $SerB^+$ prototrophy. Mapping of mutations with an NMN^+ phenotype ($R^- T^+$) was more difficult. This was done only for the Mu dJ and Mu dK insertion mutations which retained an NMN^+ phenotype. For each such mutation, a *nadI-thr* deletion mutation was constructed by recombination between these Mu d elements and *thr*::Mu dA insertions. These deletions leave the promoter end of the *nadI* gene unchanged up to the insertion site but make cells phenotypically NMN^- and Thr^-; the new phenotypes make it possible to map the insertion site. Mapping crosses were done by P22 transduction crosses, using *serB-nadI* deletion mutants ($R^- T^-$) as recipients and the *nadI-thr* deletion mutants as donors; transductants with a $Ser^+ NMN^+ Thr^+$ phenotype were selected. This selection demands an exchange between the donor and recipient deletions. (This method does not allow mapping of $R^- T^+$ point mutations.)

The deletion map is shown in Fig. 1. All $R^- T^+$ insertion mutations map at the left end of the *nadI* gene (nearest to the *serB* gene). The $R^+ T^-$ and $R^s T^-$ mutations map at the right end of *nadI* (closest to *thr*). The $R^- T^-$ mutations are scattered throughout the gene. The deletion map confirms the map order *serB-nadI*(R)-*nadI*(T)-*thr* and the conclusion that *nadI*^{s} mutations are located within the region encoding transport function.

The *nadI*(R^-) and *nadI*(T^-) mutations show complementation. Three distinct recessive phenotypes have been seen for *nadI* mutations: $R^- T^+$, $R^+ T^-$, and $R^- T^-$. This suggests

TABLE 2. Complementation test between the *nadI*($R^- T^+$) and *nadI*($R^+ T^-$) mutations

Strain	Relevant genotype	Growth on 10^{-4} M NMN	β-Galactosidase activity (U) in cells grown with indicated NA concn	
			10^{-6} M	2×10^{-4} M
TT16005	*nadB227*::Mu dA *DUP728*[(*nadI260*($R^- T^+$))*(*nadI299*($R^+ T^-$))]	+	357	5
Segregant	*nadB227*::Mu dA *nadI260*($R^- T^+$)	+	342	92
Segregant	*nadB227*::Mu dA *nadI229*($R^+ T^-$)	−	321	5

TABLE 3. *cis* effect of the *nadI260*(R^- T^+) mutation on the phenotype of *nadI*^{s}*511*

Line	Strain[a]	*nadI* allele(s)		Growth on 10^{-4} M NMN	β-Galactosidase activity (U) in cells grown with indicated NA concn	
		Copy I	Copy II		10^{-6} M	2×10^{-4} M
1	TT15998	*nadI260*(R^- T^+)	*nadI*^{s}*511*(R^s T^-)	+	5	3
2	Segregant	*nadI260*(R^- T^+)		+	361	81
3	Segregant		*nadI*^{s}*511*(R^s T^-)	−	2	2
4	TT16039	*nadI*$^+$(R^+ T^+)	*nadI260*(R^- T^+) *nadI*^{s}*5100*(R^s T^-)	+	30	4
5	Segregant	*nadI*$^+$(R^+ T^+)		+	303	6
6	Segregant		*nadI260*(R^- T^+) *nadI*^{s}*511*(R^s T^-)	−	111	57

[a] All contain a *nadB227*::Mu dA fusion.

that the transport and repression functions are distinct and each can be present without the other, but null mutations for the protein destroy both functions. If this is the case, one should see complementation between R^- T^+ and R^+ T^- mutations. The result of a complementation test is presented in Table 2. The merodiploid (TT16005) carries both the *nadI260*(R^- T^+) and the *nadI299*(R^+ T^-) alleles and is proficient for both regulation and NMN transport, while each haploid with a single allele is defective for one or the other function. This suggests that the two functions are independent. Below we present evidence that these two independent functions are provided by a single protein.

Dominance test of the two phenotypes of *nadI*^{s} mutations; superrepression is dominant and NMN utilization deficiency is recessive. Both phenotypes of *nadI*(R^s T^-) mutations have been tested for dominance in a strain carrying a tandem duplication of the *nadI* region, as described previously (32). The *nadI*(R^s T^-)/*nadI*(R^+ T^+) merodiploid strain is phenotypically R^s T^+; that is, it shows superrepression of a *nadB*::*lac* fusion but is able to transport NMN (32; Table 3). Thus, the superrepression phenotype of the *nadI*^{s} mutation is dominant to the wild-type allele, while its NMN transport deficiency phenotype is recessive.

***cis*/*trans* complementation tests indicate a single *nadI* gene.** The *cis*/*trans* complementation tests were done by using tandem *nadI* duplication strains which also carry a *nadB227*::Mu dA insertion (fusing *lac* to the *nadB* gene promoter) and a *pncA278*::Tn*10d*(Cm) insertion (to block alternative routes of NMN utilization). These outside mutations allow scoring of repression and NMN transport phenotypes. The R^- T^+ mutation chosen for this test is *nadI260*(R^- T^+); this is a nonpolar point mutation by the criterion of having no polar effect on expression of a downstream *nadI*::*lac* fusion (data not shown). Mutation *nadI511*(R^s T^-) was used to represent *nadI*(R^s) mutations. For the test of the *trans* configuration, strain TT15998 [*nadI260*(R^- T^+)/*nadI*^{s}*511*(R^s T^-)] was constructed by transducing the *nadI*^{s}*511* mutation into a *serB9 nadI260*/*serB9 nadI260* homozygous duplication strain, selecting Ser^+, and scoring for inheritance of the *nadI511*(R^s T^-) mutation. The *cis* configuration strain TT16039 [*nadI260*(R^- T^+) *nadI511*(R^s T^-)/*nadI*$^+$] was constructed by first constructing a *nadI260 nadI*^{s}*511* double mutant (see Materials and Methods). This double mutant was used to transduce a *serB9 nadI*$^+$ *pnuA*$^+$/*serB9 nadI*$^+$ *pnuA*$^+$ duplication strain; Ser^+ transductants were checked for possession of both of the donor *nadI* mutations. The structures of both *cis* and *trans* configuration strains were verified by segregation tests; all alleles were shown to be arranged as expected.

Note that in haploid strains, the superrepression phenotype of the *nadI*(R^s) mutation (Table 3, line 3) is substantially eliminated by an R^- mutation placed *cis* to R^s in a haploid strain (line 6). This is expected since superrepression requires the repressor function. The *cis*/*trans* complementation test (lines 1 and 4) demonstrates that the dominant superrepression phenotype is sixfold stronger if the R^s allele is located *cis* to a functional R^+ region (*trans* to the R^- mutation). The residual repression seen by the R^- R^s double mutant allele (line 6) is expected since the R^- mutation used does not completely eliminate repressor function (line 2); the residual dominant superrepression seen for the *cis* diploid (R^s R^-) (line 4) is presumably due to this residual repressor function of the R^- R^s allele. Some of this residual dominance could also be due to repressor subunit mixing.

Two types of *nadI*::Mu d(R^- T^+) insertions. As described above, some of the R^- insertions of Mu dJ and Mu dK are phenotypically R^- T^+. If the *nadI* region encodes a single protein, the distal portion (T) of this protein must be produced in these mutants despite the upstream insertion mutation. When examined more closely, the R^- T^+ insertions fall into two classes (types I and II).

Type I and type II insertions are similar in that they are able to suppress the auxotrophic phenotype of an *nadI*(R^s) mutation (the basis for their isolation). Both types map at the

TABLE 4. Effect of the type I and type II *nadI*::Mu d(R^- T^+) insertions on expression of the *nadB*::*lac* fusion

Strain	Relevant genotype	β-Galactosidase activity (U) in cells grown with indicated NA concn	
		10^{-6} M	2×10^{-4} M
TT16202	*nadB227*::Mu dA *nadI*^{s}*509*	3	3
TT16208	*nadB227*::Mu dA *nadI*^{s}*509 nadI554*::Mu dK(type I)	13	6
TT16206	*nadB227*::Mu dA *nadI*^{s}*509 nadI553*::Mu dK(type II)	257	163
TT16201	*nadB227*::Mu dA	247	6
TT16207	*nadB227*::Mu dA *nadI554*::Mu dK(type I)	281	9
TT16205	*nadB227*::Mu dA *nadI553*::Mu dK(type II)	307	145

TABLE 5. Effect of *serB* insertions on expression of the type I and type II *nadI*::Mu d(R^- T^+) fusions

Line	Strains	Relevant genotype	β-Galactosidase activity (U) in cells grown with indicated NA concn	
			10^{-6} M	2×10^{-4} M
1	TT15926	*nadI562*::Mu dJ(type I; $R^{\pm}T^+$)	61	59
2	TT16220	*serB1466*::Tn*10d*(Tc) *nadI562*::Mu dJ(type I)	1	1
3	TT16224	*serB1463*::Tn*10* *nadI562*::Mu dJ(type I)	25	30
4	TT15924	*nadI561*::Mu dJ(type II;R^- T^+)	5	5
5	TT16219	*serB1466*::Tn*10d*(Tc) *nadI561*::Mu dJ(type II)	3	3
6	TT16223	*serB1463*::Tn*10* *nadI561*::Mu dJ(type II)	4	4
7	TT15922	*nadI563*::Mu dJ(R^- T^-)	69	57
8	TT16218	*serB1466*::Tn*10d*(Tc) *nadI563*::Mu dJ(R^- T^-)	32	29
9	TT16222	*serB1463*::Tn*10* *nadI563*::Mu dJ(R^- T^-)	47	46

nadI locus and retain NMN transport ability. Type I insertions cause only slight relief of the superrepression phenotype of the parental R^s mutation; type II mutations appear nearly devoid of repressor function (Table 4). Type I insertions all map in the left-most deletion interval at the promoter end of the *nadI* region, while type II insertions are in the second deletion interval (Fig. 1). The NMN transport ability of type I insertions is expressed at high temperatures, while that of type II insertion is not (explained below); polar insertions in the *serB* gene show a strong polar effect on *lac* expression from type I insertions and only a weak effect on type II insertions, indicating that the *lac* operon of a type I insertion is transcribed mainly from the *serB* promoter and that of a type II insertion is expressed mainly by a promoter outside of *serB*, probably the *nadI* promoter (Table 5, line 1 to 3). Our interpretation of these results (explained in more detail below) is that type I insertions actually lie outside of the *nadI* gene (to the left of the *nadI* promoter) and reduce *nadI* expression by blocking its transcription from *serB*, while type II insertions are within the *nadI* structural gene.

Transcription from *serB* extends through the *nadI* region. A *serB* insertion mutation was found to reduce slightly the β-galactosidase level of standard *nadI*::*lac* fusions. It seems likely that this decrease is due to termination of *serB* transcripts, which may normally extend through *nadI*. Two insertion mutations *serB1463*::Tn*10* and *serB1466*::Tn*10d* (Tc) were used in these tests. The complete Tn*10* element provides a promoter for adjacent sequences, and the deletion derivative Tn*10d*(Tc) does not. If the effect of a *serB* insertion on *nadI* is due to transcription termination, rather than a simple lack of the *serB* protein, the two types of Tn*10* insertions might have distinct effects on expression of a *nadI*::*lac* fusion. Strains were constructed with combinations of *serB* alleles and various *lac* fusion alleles of the *nadI* region. The results for a standard *nadI* fusion (Table 5, lines 7 to 9) show that *nadI* transcription is reduced more by the *serB*::Tn*10d*(Tc) insert (TT16218) than it is in the strain with the *serB*::Tn*10* insertion (TT16222). For the type II fusion the same general effect is seen, but levels for this fusion are too low to permit evaluation (lines 4 to 6). These results suggest that transcription from the outward-directed promoter of *serB1463*::Tn*10* may be able to extend through the *nadI* locus. The reduced transcription of *nadI* caused by a *serB* insertion is probably not caused by a simple lack of the SerB protein. While these effects are small, the idea that transcription from *serB* might contribute to expression of the *nadI* gene is also supported by the observation that the *serB1466*::Tn*10d*(Tc) insertion partially suppresses the auxotrophic phenotype of the *nadI*^{s} mutants, while the *serB1463*::Tn*10* insertion provides no suppression. We presume that a slight reduction in level of expression of superrepressor protein allows improved expression of the *nadA* and *nadB* biosynthetic genes. Thus, type I Mu dJ insertions and *serB*::Tn*10* insertions may reduce *nadI* expression by blocking transcription of *nadI* from the *serB* promoter.

Expression of transport functions by type II *nadI*::Mu d(R^- T^+) insertion mutants is due to a promoter and initiator supplied by the *c* end of Mu. The observation of type II Mu d insertions (R^- T^+) was surprising if the *nadI* gene encodes a single bifunctional protein. How is the distal transport function expressed in these mutants? A possible answer is that the *c* end of the inserted Mu sequence might provide both a promoter and an initiation signal for expression of adjacent host sequences. This translational start site could be within Mu d sequence or could be formed at the junction of Mu d element and host sequence.

Several lines of evidence support this hypothesis. The type II insertion mutations were found at a very low frequency and were all Mu dJ or Mu dK insertions (two were found among 71 *nadI-pnuA* insertions); no Tn*10* or Tn*10d*(Tc) insertion mutants of the R^- T^+ type have been found. Transposon Tn*10* is known to have outward-directed promoters in its ends, but no translational start site has been detected (1, 9, 28). Transposon Tn*10d*(Tc) does not have the outward promoter (33). The type II Mu d insertions are all oriented with the *c* end of Mu toward the downstream part of the *nadI* locus. However, not every Mu d insertion oriented in this way expresses the transport activity. This finding suggests that some special requirements must be met to permit expression of the transport portion of the NadI protein (e.g., a Mu d element may have to be in a proper orientation, be located outside of sequences required for the transport function, and be placed in a proper reading frame).

The Mu dJ and Mu dK elements retain a short Mu sequence at their left end; the Mu *c* gene is the only intact gene included (5). Transcription of the Mu *c* gene is toward the end of the Mu genome (25), and no strong transcriptional terminator could be found in this region of Mu by computer analysis (3). Therefore, the Mu *c* gene promoter could provide transcripts that extend out of these Mu d elements. Recently, the mRNA for the Mu *c* gene has been found to be temperature sensitive (16a). This provides a way to test our hypothesis.

Type I and type II mutants were tested for growth on 10^{-4} M NMN at 30, 37, and 42°C with *nadI*$^+$ strain as the control. The type II mutants are NMN^- at 42°C, while the type I and wild-type strains transport NMN at all temperatures. We hypothesize that type I inserts merely interrupt the *serB*

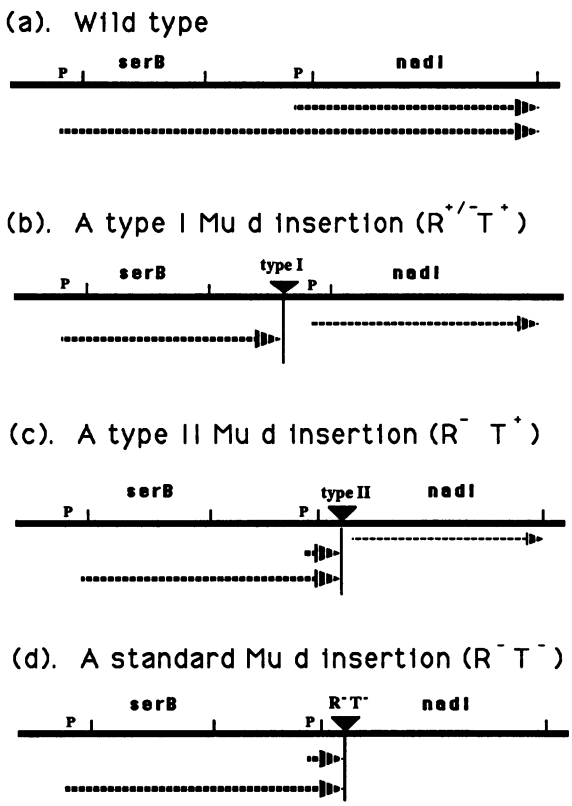

FIG. 2. Hypothesis for transcription of the *nadI* gene. (a) In a wild-type strain, *nadI* is expressed from both the *serB* promoter and the *nadI* promoter. (b) Type I insertions block *serB* transcripts and slightly reduce expression of *nadI*. (c) Type II insertions block both *serB* and *nadI* transcripts and express the downstream portion of *nadI* from its own promoter and initiator. (d) Other Mu d insertions (R^- T^-) in the same region as type II insertions may fail to express the transport portion of the *nadI* gene because they insert in an improper reading frame.

transcript and reduce *nadI* expression slightly (see above and Fig. 2). Type II insertions, we suggest, interrupt both *nadI* and *serB* transcripts, providing a downstream promoter and (if placed in a proper reading frame) an initiator to express the transport portion of the distal portion of the *nadI* gene.

The hypothesis that Mu d elements provide both a promoter and an initiator was tested in the *his* operon. A strong *rho*-dependent transcriptional terminator is located inside the *hisG* gene (8). When a Tn*10* element is placed upstream of this site, the outward Tn*10* transcripts are terminated and the next gene, *hisD*, is not expressed (8, 9). However, when a particular Mu dJ element is inserted promoter proximal to the *rho*-dependent terminator site, the strain remains $HisD^+$ (9a). This finding suggests that the Mu dJ sequence provides a promoter activity and, more important, that its transcripts seem to be translated since they can avoid termination. We believe that these Mu signals permitted isolation of the rare R^- T^+ insertions.

DISCUSSION

Mutants of the *nadI* gene were initially isolated by virtue of two distinct phenotypes. Mutations causing a defect in NMN transport were designated *pnuA*; mutations showing constitutive transcription of the *nadA* and *nadB* biosynthetic genes were designated *nadI* by us (*nadR* by Foster and co-workers [14]). In this report, we present genetic evidence that the two functions are actually provided by a single bifunctional protein. These genetic data provide functional, in vivo confirmation of conclusions of Foster and co-workers, who have found a single open reading frame in the base sequence of DNA from this region (15a).

Using mutations that cause loss of one of the functions, we can show that the two functions of the *nadI* protein can be separated. The fact that R^- T^+ mutations complement R^+ T^- mutations indicates that, at least in some mutant proteins, the two functions can act independently. However, these two functions are not always independent. Some T^- mutations in the distal region of the gene eliminate both repression and transport, and the NMN transport function can be altered so as to interfere with the repressor function. This finding initially suggested the possibility that the repressor and NMN transport functions are provided by a single protein. In addition, superrepressor mutations map at the distal (transport) end of the *nadI* gene. These point mutations cause both a dominant superrepression phenotype and a recessive NMN transport deficiency, strongly suggesting that the two distinct functions act in concert. Furthermore, double mutant strains with an R^- T^+ mutation and an R^s T^- mutation lose the superrepression phenotype (auxotrophy) but maintain their NMN transport defect, suggesting that the R^s mutation causes loss of transport activity because of a direct effect on the transport function of the *nadI* protein. We presume that *nadI*(R^s T^-) mutations cause a change in the transport portion of the protein such that the repressor portion of the molecule is active under all cellular conditions. These data alone could be explained by a bifunctional protein or by a bifunctional complex of two nonidentical polypeptides.

A *cis/trans* complementation test provided support for a single bifunctional *nadI* protein. A *nadI*^{s} mutation normally causes a superrepression phenotype (R^s) that is dominant to a wild-type allele of *nadI*. The superrepression phenotype is eliminated in a haploid strain if a *nadI*(R^- T^+) mutation is placed *cis* to the *nadI*(R^s T^-) mutation. This is expected, since repression ability is required for superrepression. If the repressor function were encoded by a gene distinct from that providing transport function, one would expect a *nadI*(R^+) allele, placed in *trans*, to completely restore the superrepression phenotype of the *nadI*(R^s T^-) *nadI*(R^- T^+) double mutant; this is not seen. For a maximal superrepression phenotype, the *nadI*(R^s) mutation must be located *cis* to a functional repressor domain. This is most easily explained by the existence of a single *nadI* protein.

Another support for the one-protein hypothesis is the existence of type II *nadI* insertions. Type II *nadI* insertions are particular Mu d insertions at the promoter-proximal end of the *nadI* region that are able to express the transport function. Other insertions of the same type of element, in the same orientation, in the same deletion interval show an R^- T^- phenotype. Type II insertions would not be expected if the *nadI* region included a single protein unless these insertions could provide both a promoter and a protein initiation site to allow expression of the distal portion of the protein. Evidence is presented that Mu d insertions can actually provide both of these signals.

The *nadI*(R^s T^-) mutations have two phenotypes, superrepression and NMN transport deficiency. The R^s phenotype is dominant and the T^- phenotype is recessive to the

wild-type allele. This supports the idea that the transport domain of the NadI protein can be altered so as to eliminate transport and qualitatively alter repressor function. More specifically, it predicts that the transport domain of the *nadI* protein may be a signal receiver as well as a component of the NMN transport system. Occupancy of the signal receiver by NAD may determine whether the repressor assumes a conformation able to bind to the *nadA* and *nadB* operator sites. The *nadI*(R^s) mutations could have changed the transport domain in such a way that it mimics the signal-received state and locks the NadI repressor domain into the DNA-binding conformation. All *nadI*(R^s) mutations lack NMN transport function even though they were isolated only by their constitutive repression phenotype; this suggests that the NadI protein cannot stimulate transport when in the repressing conformation. We suggest that the NadI protein shifts between $R^- T^+$ and $R^+ T^-$ states in response to the intracellular NAD level. This might be physiologically important for cells, since both the de novo synthesis of pyridines and NMN transport could be set in operation when the NAD level is low, and both could be turned off when the NAD level is high. In the accompanying paper (34), we present evidence that the transport function of *nadI* is not an integral part of the NMN transport system but rather serves to modulate (in response to NAD) the activity of a self-sufficient transport function provided by the *pnuC* gene product.

It is surprising that *serB* insertion mutations show polar effects on expression of *nadI*. This observation leads to the hypothesis that *serB* transcription extends out of the *serB* gene into the *nadI* gene. The *Escherichia coli serB* gene has been sequenced and is known to be transcribed clockwise (24). Just 48 bp distal to the stop codon of *serB* is a Shine-Dalgarno sequence and an open reading frame of at least 480 bp. The possibility of a larger *serB* operon structure was proposed, since no strong mRNA termination site was apparent in the determined sequence (24). The physiological significance of this operon and its relationship to *nadI* is not understood.

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