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Genetic Characterization and Regulation of the *nadB* Locus of *Salmonella typhimurium*

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The *nadB* locus encodes the first enzyme of NAD synthesis. It has been reported that this gene and *nadA* are regulated by a positive regulatory protein encoded in the *nadB* region. In pursuing this regulatory mechanism, we constructed a fine-structure genetic map of the *nadB* gene. The region appears to include a single complementation group; no evidence for a positive regulatory element was found. Several mutations causing resistance to the analog 6-aminonicotinamide mapped within the structural gene and probably cause resistance to feedback inhibition. Regulatory mutations for *nadB* were isolated. These mutants mapped far from *nadB* near the *pnuA* gene, which encodes a function required for nicotinamide mononucleotide transport. The regulatory mutations appear to affect a distinct function encoded in the same operon as *pnuA*.

The *nadB* gene encodes the first of five enzymes that act sequentially in the de novo synthesis of NAD (8, 25, 29). Mutants defective in *nadB* are auxotrophs whose nutritional requirement can be satisfied by nicotinic acid, nicotinamide, quinolinic acid, or nicotinamide mononucleotide (NMN). All of these exogenous pyridines except for quinolinic acid are converted by the salvage pathway to the biosynthetic intermediate nicotinic acid mononucleotide (NaMN) and then by the last two biosynthetic enzymes (encoded by the *nadD* and *nadE* genes) to NAD (see Fig. 1). Work on this pathway has been reviewed by Foster and Moat (12).

Expression of the *nadB* gene is regulated in response to starvation for pyridine nucleotides (6, 23, 26), and the enzyme is subject to feedback inhibition by NAD (7, 14, 25). It has been suggested that the *nadB* region encodes a positive regulatory gene (*nadR*), whose product is necessary for expression of both *nadB* and the unlinked *nadA* gene (13, 31, 32). Previously, we have described mutants that map at the *nadB* locus and that cause overproduction of NaMN; these mutants were isolated by virtue of their resistance to 6-aminonicotinamide (6AN^r) (16).

To pursue regulation of the *nadB* gene and the nature of the 6AN^r phenotype, we constructed a genetic map of the *nadB* gene and did complementation tests to define the number of functions encoded there. Constitutive mutants for *nadB* were isolated and mapped. Most affected a new gene, *nadI*, that appears to be located within the same operon as the NMN transport gene *pnuA*, which is far from *nadB*. Finding this putative regulatory gene confirms and extends results of Foster and co-workers (15).

MATERIALS AND METHODS

Strains. All strains are derivatives of *Salmonella typhimurium* LT2. The complete genotypes of strains used in this study are given in Table 1. Several derivatives of the Mu d(*lac*) phage described by Casadaban and Cohen (4) were used. These strains are defined below and have been assigned a shorthand designation to simplify genotype descriptions.

Mu dA refers to a transposition-defective derivative of the original Mu d1(Ap *lac*) phage described by Casadaban and Cohen (4) which forms operon fusions (18).

Mu dJ refers to the transposition-defective Mu d1-1734(Km *lac*) constructed by Castilho et al. (5). This phage is deleted for transposition functions and carries kanamycin resistance in place of ampicillin resistance.

Media. Nutrient broth (with 5 g of NaCl per liter; Difco Laboratories, Detroit, Mich.) was used as maximal rich medium. Minimal medium was the E-medium described by Vogel and Bonner (32) supplemented with 0.2% (wt/vol) glucose. Additional supplements, added as indicated in the text, were used at the following final concentrations: nicotinic acid and nicotinamide (2 μg/ml), 6-aminonicotinamide (6AN) and 6-aminonicotinic acid (50 μg/ml), and quinolinic acid (1 or 5 mM, as indicated). Quinolinic acid was recrystallized in cold 40% acetic acid prior to use. Quinolinic acid, when supplied at a concentration of 5 mM, was sufficient to completely satisfy the growth requirements of *nadA* and *nadB* auxotrophic mutants; with 1 mM quinolinic acid, these strains grew at a reduced rate. All other nutrient supplements were used at the final concentrations recommended by Davis et al. (10). Tetracycline was used at 10 μg/ml; ampicillin was used at 15 μg/ml in minimal medium and 30 μg/ml in rich medium. The chromogenic β-galactosidase substrate 5-bromo-4-chloro-3-indolyl-β-D-galactoside was used in solid medium at 25 μg/ml as an indicator of the expression of the *lacZ* gene of the Mu d(*lac*) phages.

Identification of nonsense mutations in the *nadB* and *nadI* genes. Suppressible mutations were identified by use of strains containing the *nadB103* deletion and one of the following suppressor mutations: *supD* (strain TT10087), *supE* (strain TT10086), or *supU* (strain TT10085). These strains were used as recipients in transductional crosses, and simple *nadB* auxotrophs were used as donors. These donors were unable to give prototrophic recombinants when the *nadB103* deletion in a wild-type background (*sup*$^+$) (strain TT9922) was used as a recipient. Donors with amber or UGA-suppressible *nadB* mutations, however, gave prototrophic recombinants when the recipient carried a suppressor that was capable of correcting the defect of the donor allele. The *nadB* mutants identified as UGA or amber (UAG) are shown in Table 2.

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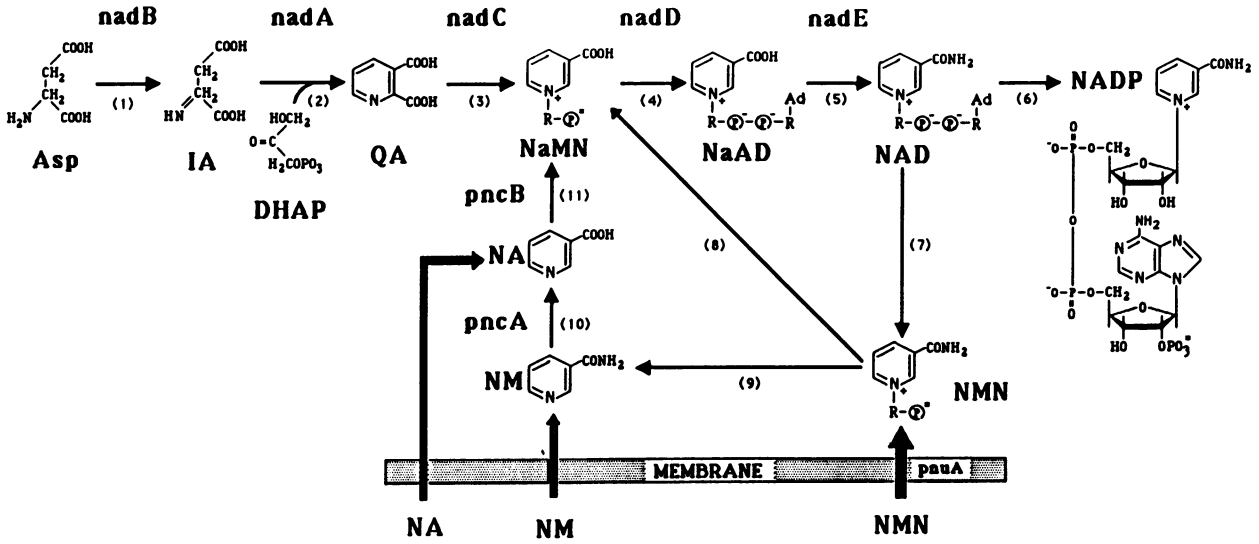

FIG. 1. The NAD metabolic pathway of *S. typhimurium*. The following enzymes are included: L-aspartate (Asp) oxidase (step 1); quinolinic acid (QA) synthetase (step 2); quinolinic acid PRPP phosphoribosyl transferase (step 3); NaMN adenylyl transferase (step 4); NAD synthetase (step 5); NAD kinase (step 6); DNA ligase (step 7); nicotinamide mononucleotide (NMN) deamidase (step 8); NMN glycohydrolase (step 9); nicotinamide (NM) deamidase (step 10); nicotinic acid (NA) PRPP phosphoribosyl transferase (step 11). Abbreviations: DHAP, dihydroxyacetone phosphate; IA, iminoaspartate; PRPP, 5-phosphoribosyl-1-PP_i. Genetic loci corresponding to enzymatic steps are indicated above the reaction arrow.

Amber mutations in *nadI* were identified by transduction crosses with recipient strains carrying a *nadI* mutation and a *nadB*::Mu dA fusion. The donor strain (TT2069) carried a Tn*10* insertion that was 100% linked to a *supD* amber suppressor. When mutations for tetracycline resistance were selected, every transductant received an amber suppressor; these transductants were then scored for correction of their *nadI* phenotype by testing their ability to repress β-galactosidase expression in response to a high exogenous concentration of nicotinic acid. The following alleles showed correction: *nadI257*, *nadI258*, *nadI260*, *nadI265*.

Selection of Tn*10*-generated deletions. Strains TT6581 and TT6579, which carry Tn*10* insertions near the *nadB*$^+$ gene, were subjected to selection for tetracycline-sensitive derivatives as described by Bochner et al. (3) and Maloy and Nunn (22). We found that this selection works much better at 40°C, and most selections were performed at this temperature.

Enzyme assays. β-Galactosidase was assayed in Mu d-containing strains as described by Miller (24) by using the CH_3Cl-sodium dodecyl sulfate permeabilization procedure. The β-galactosidase activity is reported as nanomoles per minute per optical density unit (650 nm).

Fine-structure deletion mapping. Deletion mapping was done by transductional crosses mediated by P22 (HT105, *int-201*). A fresh overnight culture of recipient cells (10^9 cells per ml) was concentrated 10-fold by centrifugation and then infected at a multiplicity of 10 with a transducing lysate. The suspension of infected cells was plated on selective medium (0.1 ml per plate) and incubated for 3 days before it was scored. A wildtype donor typically yielded 10^5 recombinant (NAD^+) colonies per plate under these conditions. A cross was scored as negative if no recombinants were seen on five plates.

RESULTS

A genetic map of the *nadB* region. Using P22-mediated transduction crosses, we constructed a genetic map of the *nadB* region (Fig. 2). The map was constructed by using Tn*10*-generated deletion mutants as recipients and point mutations obtained by local mutagenesis as donors. The isolation of these mutant types is also described below. The resolution of the map is such that a failure to recombine represents at least 5×10^5-fold fewer recombinants than would have been seen if a wild-type donor strain had been used. Previous results (20) have shown that this sensitivity can resolve mutations separated by on the order of 10 base pairs.

Tn*10*-generated deletions and their endpoint distribution. The deletions presented in Fig. 2 were derived from a strain carrying a Tn*10* insertion (*zfi-789*::Tn*10*) near the *nadB* gene (strain TT6581). This strain was subjected to selection against tetracycline resistance as described by Maloy and Nunn (22). Survivors were screened for nicotinamide auxotrophy. Each deletion presented arose independently.

The deletion endpoint distribution was nonrandom. Of 29 deletions that were isolated initially, 9 ended at one hot spot, and the rest had endpoints very near that hot spot. The three shortest deletions were isolated from an additional set of 100 Tn*10*-generated *nadB* deletions; they were the only ones that recombined with point mutation *nadB58*. No deletions subdivided the large cluster of mutations at the left side of the map, even though recombination tests between these points demonstrated that a large number of sites are represented. The deletion distribution suggests that endpoints are preferentially at the hot spot, but occur with progressively decreasing probability at sites removed from the hot spot. The results suggest that a sequence at the hot spot may contribute to the frequency of Tn*10*-generated deletions whose endpoint is slightly removed from that site.

Auxotrophic *nadB* point mutations. The point mutations presented in Fig. 2 were isolated by local mutagenesis as outlined above. All mutations causing simple auxotrophy were induced by hydroxylamine mutagenesis. A classification of these mutations is presented in Table 2. The nonsense mutations were identified as described above. All auxo-

trophic mutations in Table 2 that are not identified by number on the map in Fig. 2 mapped within the first deletion interval.

Analog-resistant *nadB* mutations. Growth of *Salmonella* is strongly inhibited by the nicotinamide analog 6AN. This analog is apparently converted to the analog of NAD which is toxic to growth (Fig. 1). Mutations which prevent conversion of 6AN to the analog of NAD cause resistance to the analog (12, 16). Of mutants resistant to 6AN, most affected *pncA* and *pncB*, the genes whose products are responsible for converting exogenous 6AN to 6-aminonicotinic acid mononucleotide. One class of analog resistance mutation mapped at the *nadB* locus, however, and was presumed to owe its resistance to unregulated de novo synthesis of NAD (16). We isolated two types of 6AN^r mutations at the *nadB* locus by local mutagenesis, which was performed by the same method used for the generation of *nadB* auxotrophs described above. These mutants are listed in Table 2. One type of *nadB* mutant showed simple resistance and grew in the presence of 6AN or the nicotinic acid analog 6-aminonicotinic acid; these mapped at *nadB* but were not placed on the fine-structure map. The second type was analog resistant at 30°C, but showed pyridine auxotrophy at 40°C. These last mutations, because of their auxotrophic phenotype at high temperatures, could be deletion mapped with high resolution. Their map positions are indicated on the genetic map in Fig. 2.

TABLE 1. Strains used in this study

Strain	Genotype[a]	Source[b]
TR4602	*trpR520*	Elias Balbinder
TR4604	*serB9 trpR520*	Elias Balbinder
TR5129	DUP55 (*argB*$^+$ *hisD*$^+$) (*argB*$^+$ *his-63*)	
TR5985	*nadB52 purC7 proA46 ilv-405 rha-461* StrR m10 *fla-56 fim*	John Foster
TR5988	*nadA56 pncA15 trpA49 pnuA11* (T$^-$ R$^+$)	John Foster
TR6416	*nadD157*	
TR6450	*nadB63 pncB9 trpA49*	John Foster
TR6509	*nadB51 pncA15 trpA49*	John Foster
TT399	*nadB214*::Tn*10*	
TT2069	*hisC527*(Am) *leu-414*(Am) *supD zeb-607*::Tn*10*	
TT6579	*zfi-787*::Tn*10*	
TT6581	*zfi-789*::Tn*10*	
TT7247	*zbe-1023*::Tn*10*	
TT7674	*pncA212*::Mu dA	
TT7692	*hisD9953*::Mu dA (Lac$^+$)	
TT7693	*hisF9954*::Mu dA (Lac$^+$)	
TT7694	*hisE9957*::Mu dA (Lac$^-$)	
TT7751	*supD*501 *leu-414* (Am) DUP403 [(*nadD*$^+$ *zbe-1028*)*Tn*10* *(*zbe-1023 nadD157*)]	K. Hughes
TT8785	*nadA219*::Mu dA (Lac$^+$)	
TT8786	*nadC220*::Mu dA (Lac$^+$)	
TT8790	*nadB224*::Mu dA (Lac$^-$)	
TT8791	*nadB225*::Mu dA (Lac$^-$)	
TT8792	*nadB226*::Mu dA (Lac$^-$)	
TT8793	*nadB227*::Mu dA (Lac$^+$)	
TT9835	*nadB227*::Mu dA *nadI241*::Tn*10*	
TT9836	*nadA219*::Mu dA *nadI242*::Tn*10*	
TT9839	*nadA219*::Mu dA *nadI241*::Tn*10*	
TT9840	*nadB227*::Mu dA *nadI242*::Tn*10*	
TT9922	*nadB103* (no suppressors)	
TT10078	*nadA219*::Mu dA *nadB214*::Tn*10*	
TT10079	*nadA219*::Mu dA *nadB103*	
TT10080	*nadA219*::Mu dA *nadB111*	
TT10081	*nadA219*::Mu dA *nadB55*	
TT10082	*nadA219*::Mu dA *nadB133*	
TT10083	*nadA219*::Mu dA *nadB137*	
TT10084	*nadA219*::Mu dA *nadB141*	
TT10085	*nadB103 zhb-736*::Tn*10 supU1283*	
TT10086	*nadB103 zbf-100*::Tn*10 supE*	
TT10087	*nadB103 zeb-607*::Tn*10 supD*	
TT10158	*serB1463*::Tn*10 nadI325 pncA15 nadA219*::Mu dA	
TT10178	*serB9 nadI242*::Tn*10*	

[a] Nomenclature is as described by Demerec et al. (11), Chumley et al. (9), and Schmid and Roth (27).

[b] Except for the strains whose source is designated, strains were either constructed in the course of this work or were obtained from the *Salmonella* strain collection, University of Utah.

TABLE 2. Characterization of *nadB* point mutations

Mutant type	*nadB* allele no.
Simple auxotrophs	51, 52, 53, 54, 55, 57, 58, 59, 60, 61, 62, 63, 64, 65, 66, 68, 71, 72, 73, 74, 75, 76, 77, 78, 79, 80, 82, 83, 84, 87, 89, 91, 93, 94, 96, 97, 98, 99
UGA nonsense auxotrophs	81, 88, 92
UAG nonsense auxotrophs	56, 100
Leaky auxotrophs	67, 69, 70, 85, 90, 95
Thermosensitive auxotrophs	164, 165, 166, 167, 168, 169, 170, 171, 172, 173, 174, 175, 176, 177, 178, 179, 180, 181, 182, 183, 184, 185, 186
Resistant to 6AN	131–143
Resistant to 6AN at 30°C and auxotrophic at 40°C	200–208

Placing the promoter on the *nadB* genetic map. The promoter for *nadB* is located at the left side of the gene, as it appears in the genetic map (Fig. 2). This was determined by observing polarity effects on *nadB*::*lac* fusions. (These fusions will be discussed below.) Double mutants were constructed that carried the *nadB214*::Tn*10* (located in the leftmost deletion interval) and the *nadB227*::Mu dA insertion (located in the eighth deletion interval). The parent Mu dJ insertion expressed its *lac* operon from the regulated *nadB* promoter. The double mutant did not express *lac*. This suggests that transcription proceeds from left to right and is blocked by the Tn*10* insertion. Similarly, a double mutant carrying the deletion mutant *nadB111* and the same *nadB 227*::Mu dJ also failed to express the *lac* operon.

Orienting the *nadB* gene in the chromosome. The availability of *nadB*::Mu d(*lac*) fusions (described below) makes it possible to determine the direction of transcription of the *nadB* gene in the chromosome by the method described by Hughes and Roth (19). The basic strategy is to generate tandem duplications in transduction crosses by using as the donor phage a mixture of P22 lysates grown on two different Mu d insertion mutants. Two different Mu d prophages are transduced simultaneously into a single recipient cell by different transduced fragments and recombine to form a novel duplication join point which is inherited to generate the corresponding duplication. These duplication-carrying transductants can be identified because they show neither of the auxotrophic requirements of the two donor insertion mutants. This occurs only if the two donor Mu d prophages are in the same orientation on the chromosome. Thus, if a duplication is formed, it can be concluded that the two Mu d

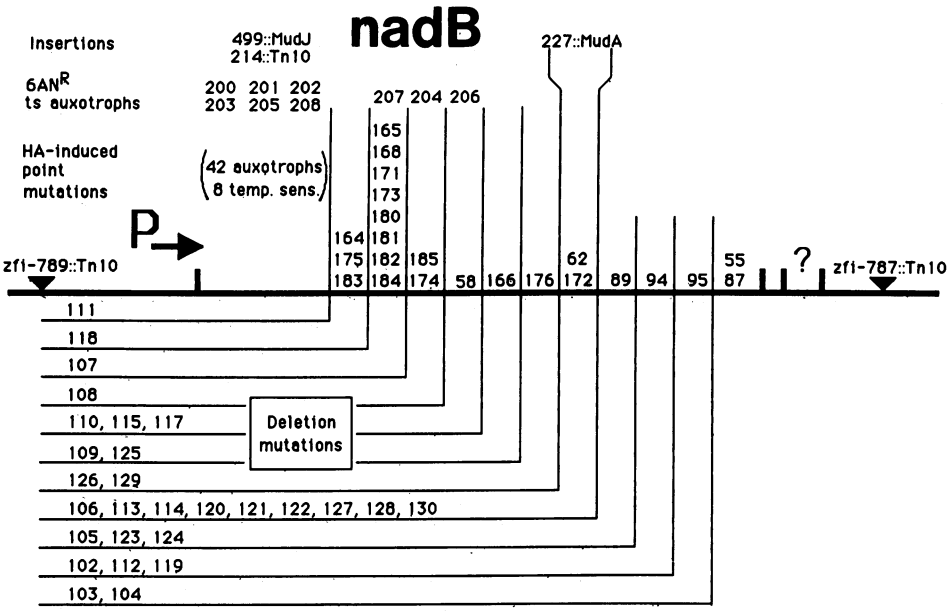

FIG. 2. Deletion map of the *nadB* region. Deletion mutations, listed below the heavy horizontal line, were all isolated as tetracycline-sensitive derivatives of the insertion mutant *zfi-789*::Tn*10*. The type of point and insertion mutants mapped are indicated at the left, above the horizontal line. Transcription was from left to right. P, Promoter location; HA, hydroxylamine.

prophages used must be inserted in the same orientation in the chromosome.

To determine the direction of *nadB* transcription, various *his*::Mu d and *nadB*::Mu d insertions were tested for their ability to generate *nadB-his* duplications. Duplication events between *his* and *nadB* were only seen when Mu d insertions with the same Lac phenotype were tested (Table 3). The results presented above indicate that *his* and *nadB* genes are both transcribed in the same direction. Since the *his* operon is known to be transcribed clockwise, *nadB* must also be transcribed clockwise. Since the direction of transcription of the *nadB* genetic map is known (see text above and Fig. 2), the *nadB* map can be oriented in the chromosome. Thus, the left side (with the promoter) of the map in Fig. 2 must be located counterclockwise of the *nadB* gene (closest to *his*) in the chromosome.

The *nadB* region is a single complementation unit. Results of previous work with *Escherichia coli* have suggested that *nadB* mutations fall into two groups: one that is defective in only *nadB* function and one that is defective in both *nadB* and *nadA* function. Since the *nadA* gene is unlinked to *nadB*, it was suggested that a positive regulatory element (*nadR*) might be encoded in the *nadB* region (13, 14, 30, 31). To test this possibility, we performed complementation tests between auxotrophic mutations at the *nadB* locus. In these tests we used the temperature-sensitive auxotrophic 6AN^r mutations. We tested the ability of these temperature-sensitive mutations to complement a series of standard *nadB* point mutations, including one near the right end of the map and five from the cluster of mutations at the left end of the map. All pairs of mutations tested failed to complement, suggesting that they belong to a single complementation unit.

The tests described above were done by using a chromosomal tandem duplication which includes the region from *argA* (minute 61) to *his* (minute 44) and whose only functional *hisD* gene is located at the duplication join point (see Fig. 3, line 1). Background information on the origin and genetic behavior of such duplications has been published previously (1, 2). The *nadB* mutations to be tested were introduced (by using a linked Tn*10* insertion as a selective marker) into a strain carrying a *his* deletion mutation too large to be repaired by transduction; this generated strains such as that in Fig. 3, line 2. Phage on the duplication strain (Fig. 3, line 1) was used to transduce the *nadB his* (deletion) recipient selecting HisD$^+$ (growth on histidinol). Simple repair of the recipient *his* deletion by donor *hisD*$^+$ material was impossible. Coinheritance of *hisD*$^+$ and the duplication join point (diagrammed in Fig. 3, line 2) regenerated the duplication in the recipient strain and gave rise to a HisD$^+$ transductant. These transductants were diploid for the *nadB* locus and had the recipient allele in both copies of the duplication (Fig. 3, line 3). Selection for growth on histidinol (HisD$^+$) maintained the duplication state. Into each of the *nadB* mutant duplication strains a temperature-sensitive

TABLE 3. Transcriptional orientation of *nadB*[a]

nadB::Mu dA donor	Prototrophic duplications formed in crosses with *nadB*::Mu dA and[b]:			
	hisD9953 (Lac$^+$)	*hisF9954* (Lac$^+$)	*hisE9957* (Lac$^-$)	None
nadB224 (Lac$^-$)	−	−	+	−
nadB225 (Lac$^-$)	−	−	+	−
nadB226 (Lac$^-$)	−	−	+	−
nadB227 (Lac$^+$)	+	+	−	−
None	−	−	−	−[c]

[a] For each transduction cross, a mixture of P22 lysates (one grown on a *nadB*::Mu dA strain and one on a *his*::Mu dA strain) was used as the donor; the recipient strain was LT2 (wild type). Ampicillin-resistant recombinants were selected and scored for prototrophy (possession of a duplication). The strains used as donors in these crosses are listed in Table 1.

[b] A + indicates that this combination of donors yielded prototrophic (duplication-carrying) Amp^r transductants. A − indicates that all Amp^r colonies were auxotrophic for either nicotinamide or histidine.

[c] No Amp^r colonies arose.

nadB allele was introduced by selection for *nadB*$^+$ at 30°C. This generated a diploid with the genotype *nadB*/*nadB*(Ts).

By testing the ability of this diploid to grow at 40°C it could be determined whether the introduced temperature-sensitive mutation complemented the *nadB* allele that was present in the diploid. Five standard *nadB* auxotrophic point mutations (*nadB62*, *nadB63*, *nadB71*, *nadB83*, *nadB99*) were tested against nine *nadB* temperature-sensitive alleles (*nadB200* to *nadB208*). No pair showed complementation. One of the standard *nadB* alleles tested was located at the right side of the map; the other five were located in the large cluster of points at the left side of the map. Six of the temperature-sensitive mutations were in the large cluster at the left; the other three were within three different deletion intervals near the middle of the gene. For each pair, the structure of the diploid was verified by allowing the duplication to segregate by recombination between copies, giving rise to HisD$^-$ haploid segregants. These were then scored for their pyridine auxotrophy phenotype. Each diploid that was used to infer a lack of complementation gave rise to a mixture of standard and temperature-sensitive pyridine auxotrophs among its HisD$^-$ haploid segregants, thus demonstrating that both *nadB* alleles were, in fact, present in the merodiploid. All the simple auxotrophic *nadB* mutations were shown to be recessive by the use of wild-type donors in the tests described above. The auxotrophic phenotype of the thermosensitive 6AN^r mutations were shown to be recessive at 40°C (see below).

Mutations that cause dominant analog resistance and recessive auxotrophy. We have reported previously (16) mutations at the *nadB* locus that cause a dominant resistance to 6AN. As mentioned above, we have found *nadB* mutants that are analog resistant at 30°C and pyridine auxotrophs at 42°C. We have tested the dominance of both phenotypes of these *nadB* mutations. In all cases analog resistance (tested at 30°C) was dominant and auxotrophy (tested at 42°C) was recessive to the wild-type *nadB* allele.

Transducing lysates were prepared on strains carrying an analog resistance mutation linked 65% to a Tn*10* insertion near *nadB*. Each lysate was used to transduce tetracycline resistance into a duplication strain carrying two wild-type alleles of the *nadB* locus (strain TR5129). In each case, approximately 65% of the Tet^r transductants were resistant to 6AN, demonstrating that the introduced resistance mutation was dominant over the wild-type copy of *nadB*. In the case of temperature-sensitive analog resistance mutations, these tests were done at 30°C. Since resistance was apparent even in the presence of a wild-type *nadB* allele, both simple and thermosensitive resistance phenotypes (tested at 30°C) were inferred to be dominant. The structure of each diploid was verified by finding a mixture of *nadB*$^+$ and *nadB* (6AN^r) or *nadB* (6AN^r[Ts]) segregants among the HisD$^-$ haploid segregants.

The diploids involving temperature-sensitive 6AN^r alleles *nadB*$^+$/*nadB* (6AN^r[Ts]) were also tested at 40°C. At this temperature, the strains remained prototrophic; thus, the auxotrophy of the temperature-sensitive mutations is recessive. All such diploids became analog sensitive at 40°C. Thus, they lose the dominant resistance phenotype as they lose the mutant enzymatic activity. This suggests that the resistance conferred by these mutations depends on the enzymatic activity of the mutant protein. This is consistent with the idea that 6AN^r is caused by a structural alteration of the *nadB* enzyme (e.g., resistance to feedback inhibition). In addition, several of these mutations (*nadB207*, *nadB204*, nadB206) mapped within the structural gene, as defined by the map shown in Fig. 2. The above dominance tests were made for the simple resistance mutations *nadB131* to *nadB140*) and for the thermosensitive resistance mutations *nadB203* to *nadB207*; the results in all cases were as described above.

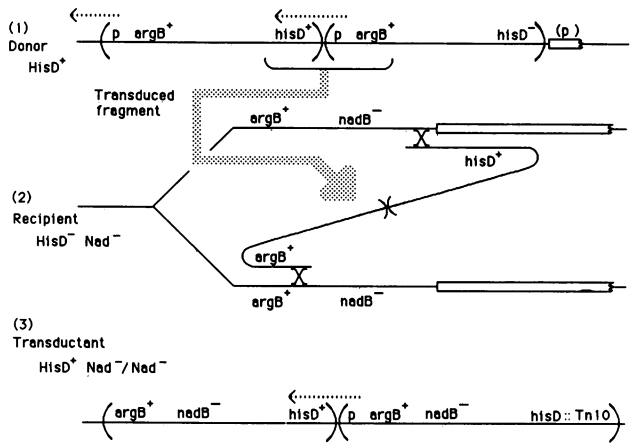

FIG. 3. Construction of merodiploids for complementation. Complementation tests employed tandem chromosomal duplications generated by the cross described above. The donor had a duplication carrying a *hisD* gene near the join point; this copy of *hisD* was the only functional *hisD* gene and was expressed because it fused to a foreign promoter that transcribed across the join point. The second copy of *hisD* was not expressed because it lacked a promoter. Isolation and characterization of this duplication have been described previously (1, 2). Since the only functional copy of the *hisD* gene in the donor strain was at the join point, use of the duplication strain as a donor to repair a recipient *hisD* deletion resulted in inheritance of the join point, as diagrammed. The HisD$^+$ transductants carried a duplication that was identical in extent to that of the donor, but donor material from only the join point region was inherited; the majority of the duplicated material (including the *nadB* gene) was of recipient origin. These duplications were used in complementation tests by introducing various *nadB* markers, as described in the text.

Isolation and behavior of *lac* operon fusions for *nadA* and *nadB*. For the experiments in which we isolated and determined the behavior of *lac* operon fusions for *nadA* and *nadB* we used Mu dA, a transposition-defective derivative of the original Mu dl(Ap *lac*) (18). The insertions used to characterize regulation were *nadA219*::Mu dA and *nadB227*::Mu dA. Regulation of β-galactosidase in these fusion strains is presented in Table 4. Quinolinic acid is the intermediate in the NAD pathway that is produced by the combined activities of the *nadB* and *nadA* gene products (Fig. 1). Since quinolinic acid is poorly assimilated, growth of *nadA* or *nadB* mutants on this intermediate is slow and the growth rate is limited by the decreased rate of NAD synthesis. Growth under these conditions of pyridine limitation resulted in high levels of transcription of both *nadA* and *nadB* genes, as judged by the level of β-galactosidase present in the operon fusion strains (Table 4). The addition of nicotinamide, which is efficiently assimilated, resulted in repression of transcription of both genes (*nadA* and *nadB*).

***nadB* mutations do not affect transcription of *nadA*::*lac* fusions.** Since results of previous work have suggested the possibility that *nadB* mutations might alter expression of the *nadA* gene (13, 14, 30, 31), we tested directly the effect of various *nadB* mutations on the transcription of *nadA*::*lac* operon fusions. This was done by constructing double mutants with both the *nadA*::Mu dA operon fusion and the

TABLE 4. Expression of *nadA*::Mu d and *nadB*::Mu d fusions

Strain	Relevant genotype	Minimal medium supplement[a]	β-Galactosidase activity[b]
TR8785	*nadA219*::Mu dA	Qa	247
		Nm + Qa	5.4
TR8793	*nadB227*::Mu dA	Qa	185
		Nm + Qa	3.9
TR9836	*nadA219*::Mu dA *nadI242*::Tn*10*	Nm + Qa	273
TR9839	*nadA219*::Mu dA *nadI241*::Tn*10*	Nm + Qa	260
TR9840	*nadB227*::Mu dA *nadI242*::Tn*10*	Nm + Qa	218
TR9835	*nadB227*::Mu dA *nadI241*::Tn*10*	Nm + Qa	219

[a] Cells were grown in minimal medium with glucose as the carbon source. Nicotinamide (Nm) was added where indicated at a concentration of 2 μg/ml; quinolinic acid (Qa) was added where indicated at a concentration of 1 mM.
[b] Activity is expressed in nanomoles of product per minute per optical density unit of permeabilized cells.

nadB mutation to be tested (Table 5). Included in these tests were a deletion which removed all but two mutation sites of the *nadB* locus (*nadB103*), a *nadB*::Tn*10* insertion mutation, a point mutation that was in the rightmost interval of the map (*nadB55*), the shortest deletion that removed the left end of the gene (*nadB111*), and three simple analog resistance mutations (*nadB133*, *nadB137*, *nadB141*). None of these *nadB* mutations had a significant effect on the expression or regulation of the *nadA* gene. Thus, we found no evidence that any *nadB* mutant lacks a positive regulatory protein that acts on the *nadA* gene.

Isolation of potential regulatory mutants for *nadA* and *nadB*. Both *nadA*::Mu dA and *nadB*::Mu dA insertions formed light-colored colonies on MacConkey-lactose indicator medium containing nicotinamide. The repressed level of transcription from the *nadA* and *nadB* promoters apparently produces insufficient levels of β-galactosidase to permit the acid production which indicates a Lac^+ phenotype on this medium. Following mutagenesis of the *lac* fusion strains with either Tn*10* or diethylsulfate, however, red colonies were observed on MacConkey-*lac*-nicotinamide medium at a frequency of approximately 10^{-3} to 10^{-4} (number of red colonies per total number of colonies). Enzyme assays verified that these mutants produced elevated levels of β-galactosidase (in the range of 150 to 250 U of β-galactosidase). All constitutive mutations tested (including members of all the classes discussed below) had an equivalent effect on both the *nadA* and *nadB* genes, suggesting that these two genes are subject to regulation in response to the same regulatory mechanism. Data for two Tn*10* insertion mutations are presented in Table 4.

Classification of constitutive mutations. The constitutive mutations have been classified into three groups based on results of genetic mapping. Of 32 diethylsulfate-induced constitutive mutations, 26 showed close linkage to *serB*. Mutations in this region were designated *nadI*. Holley et al. (15) have also reported the existence of regulatory mutants for *nad* in this region.

Three of the remaining six diethylsulfate-induced constitutive mutations appeared to affect the *nadD* gene. All three showed transductional linkage to a Tn*10* insertion near *nadD* (*zbe-1023*::Tn*10*) (20). The three mutations showed 98% transductional linkage to a *nadD* temperature-sensitive mutation (*nadD157*). Results of previous work (17) have shown that leaky *nadD* mutations are quite common and cause an accumulation of the intermediate NaMN. It is likely that such mutations would also limit the pool of NAD in the cells. We propose that the constitutive phenotype of the *nadD* mutations described here is a physiological response to a reduced level of NAD (or NADP). Consistent with this hypothesis is the observation that other alleles of *nadD* (isolated as resistant to 6AN) also show constitutive expression of *nadA* and *nadB* operon fusions (17). Thus, the constitutive phenotype of these mutations is probably a response to a limitation of the end product and not to a defect in the regulatory mechanism.

The three remaining diethylsulfate-induced constitutive mutants mapped at neither the *nadI* nor the *nadD* locus. They are not characterized in detail here, but they were all closely linked to each other.

Characterization of the *nadI* mutations. As mentioned above, mutations classified as *nadI* proved to be closely linked to *serB* in P22-mediated crosses. Foster has shown that this region of the genetic map includes the gene *pnuA* which is involved in transport of NMN (21). We tested the new regulatory mutants for their ability to transport NMN (see Materials and Methods). This test allowed us to divide the *nadI* mutations into two classes, according to whether or not they possessed NMN transport ability. Of 26 diethylsulfate-induced *nadI* regulatory mutants, 16 were defective for NMN transport (T^- R^-) and 10 were able to transport NMN (T^+ R^-). Thus, some *nadI* and *pnuA* mutants share one aspect of their phenotype. For simplicity in presentation, we refer to all mutations in this region as *nadI* and describe them by phenotype. Mutants described as *nadI* T^- R^- were defective in both transport and regulation; mutants described as *nadI* T^+ R^- were defective only in regulation. Mutants defective only in transport are referred to as *nadI* T^- R^+.

Mapping of the *nadI* region. The *nadI* constitutive mutations were mapped vis à vis nearby markers by three-point transduction crosses. In addition to the *serB* gene, which was shown earlier to be linked to *nadI*, the region is known to include the *thrA* and *trpR* loci. Results of these crosses (Table 6) indicate that the two *nadI* mutants tested both lie

TABLE 5. Effect of *nadB* mutants on a *nadA*::Mu d

Strain genotype	Strain	β-Galactosidase activity[a] for cells grown on:	
		Qa	Qa + Na
nadA219::Mu d	TT8785	248	6.9
nadA219::Mu d *nadB103* (deletion)	TT10079	198	3.6
nadA219::Mu d *nadB214*::Tn*10*	TT10078	241	7.1
nadA219::Mu d *nadB55* (point)	TT10081	154	6.5
nadA219::Mu d *nadB111* (deletion)	TT10080	261	3.5
nadA219::Mu d *nadB133* ($6AN^r$)	TT10082	132	8.1
nadA219::Mu d *nadB137* ($6AN^r$)	TT10083	199	6.8
nadA219::Mu d *nadB141* ($6AN^r$)	TT10084	137	6.6

[a] Cells were grown on minimal medium with either 1 mM quinolinic acid (Qa) or 1 mM quinolinic acid plus 2 μg of nicotinic acid (Na) per ml. Units of activity are nanomoles of product per minute per optical density unit of permeabilized cells.

TABLE 6. Three-factor crosses involving *nadI*

Cross	Strain	Genotype of parents[a]				Genotype of recombinants				Recombinants	
		serB	*nadI*	*trpR*	*thr*	*serB*[b]	*nadI*	*trpR*	*thr*	No.	Type[c]
Cross 1 (*nadI277*::Tn*10*)	Donor	+	−	+	+	+	−	+	+	181	Donor
	TT9836					+	−	−	+	178	
	Recipient	−	+	−	+	+	+	−	+	135	
	TT4604					+	+	+	+	0	4 CO
Cross 2 (*nadI272*::Tn*10*)	Donor	+	+	−	+	+	+	−	+	384	Donor
	TR4602					+	+	+	+	74	
	Recipient	−	−	+	+	+	−	+	+	27	
	TT10178					+	−	−	+	15	4 CO
Cross 3 (*nadI272*::Tn*10*)	Donor	+	+	+	−	+	+	+	−	98	Donor
	thrA10					+	+	+	+	373	
	Recipient	−	−	+	+	+	−	+	+	28	
	TT10178					+	−	+	−	1	4 CO
Cross 4 (*nadI325*)	Donor	+	+	+	−	+	+	+	−	47	Donor
	thrA10					+	+	+	+	376	
	Recipient	−	−	+	+	+	−	+	+	76	
	TT10158					+	−	+	−	1	4 CO
Cross 5 (*nadI325*)	Donor	+	+	−	+	+	+	−	+	397	Donor
	TR4602					+	+	+	+	44	
	Recipient	−	−	+	+	+	−	+	+	53	
	TT10158					+	−	−	+	5	4 CO

[a] Mutations are listed from left to right in the order inferred from the results of these crosses.
[b] In all crossses, organisms with the SerB$^+$ phenotype were selected.
[c] Donor type recombinants are designated donor. The recombinants requiring four crossover events, if the inferred order is correct, are designated 4 CO.

between *serA* and *trpR*. One of the mutations (*nadI242*::Tn*10* T$^-$ R$^-$) was defective in both NMN transport and regulation of *nadA* and *nadB*. Another mutation tested (*nadI*325 T$^+$ R$^-$) was defective only in regulation. Both mutations lay between *serB* and *trpR*; this is also the location of *pnuA* mutations (21, 28). A map of the general area, including transductional linkages inferred from these crosses, is presented in Fig. 4. The relative order of the three *nadI* mutations is merely a proposal since the linkage data are not sufficient to establish positions. We propose this order since several *nadI* (amber) regulatory mutations retained transport function, and thus, the regulation function is likely to be downstream in the operon; transcription is known to proceed from left to right (Ning Zhu and John Roth, unpublished data).

Local mutagenesis of the *pnuA-nadI* region. To clarify the relationship between the NMN transport function and the regulatory function of this region, we isolated a set of mutations following localized mutagenesis of the *serB* region. A transducing lysate was grown on a *serB*::Tn*10* insertion mutant and mutagenized with hydroxylamine, as outlined in Materials and Methods. The lysate was used to introduce the mutagenized *serB* region into an unmutagenized recipient carrying a *pncA* mutation and a *nadA*::Mu dA *lac* fusion both unlinked to *serB*; Tet^r transductants were selected. The resulting transductants were scored for ability to use NMN as a pyridine source and for the ability to repress the *lac* operon fused to *nadA*. All three possible mutant types were recovered. (For purposes of clarity, we have assigned the *nadI* designation to all mutations until the relationship with the *pnuA* gene can be clarified.)

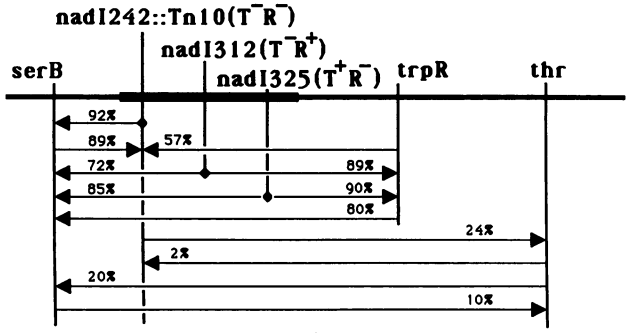

FIG. 4. Genetic linkages in the *nadI* region. All linkages are given as cotransduction frequencies determined in P22-mediated crosses. The selected marker is indicated by the arrowhead. The order of markers within the *nadI* region is a best guess; the available linkage data are not sufficient to establish this order.

Of a total of 57 mutants isolated, 31 were defective only in NMN transport (T$^-$ R$^+$), 20 were defective in both transport and regulation (T$^-$ R$^-$), and 6 were defective only in regulation (T$^+$ R$^-$). This result is consistent with the existence of two genes in a single operon, with some mutations in the upstream gene exerting polar effects on the expression of the other mutations. The results are also consistent with the existence of a single gene that encodes a bifunctional protein. More complex explanations are also possible.

Both of the Tn*10* insertions that were isolated as regulatory mutants were also defective for NMN transport. Among the diethylsulfate mutations discussed above were four amber mutations. Two of these (*nadI257* and *nadI258*) lacked both transport and regulation functions; the other two amber mutants (*nadI260* and *nadI265*) were defective only in regulation. While these results do not definitively distinguish between the possibilities described above, they suggest that the transport function may be encoded by promoter sequences located proximal to the regulatory function. A summary of constitutive mutants is presented in Table 7. A

TABLE 7. Constitutive mutations

Isolation method	Allele no. of the following *nadI* mutations[a]:			Other constitutive mutations
	nadI (T^- R^-)	*nadI* (T^+ R^-)	*nadI* (T^- R^+)	
1. Screening Tn*10* insertions for *nadB* constitutivity	241, 242			
2. Diethylsulfate mutagenesis and screening for *nadB* constitutivity	257(Amb), 258(Amb), 262, 263, 264, 266, 270, 271, 273, 275, 277, 279, 280, 284, 286, 288	260(Amb), 261, 265(Amb), 267, 269, 272, 276, 278, 283, 287		*nadD268*, *nadD274*, *nadD285*, *nad-259*, *nad-281*, *nad-282*
3. Local mutagenesis of *serB* region, screen for both NMN transport and regulatory defects	320, 321, 322, 323, 326, 327, 329, 330, 334–345	324, 325, 328, 331, 332, 333	289–319	

[a] For isolation method 1, there were two *nadI* T^- R^- mutations. For isolation method 2, there were 16 *nadI* T^- R^- mutations, 10 *nadI* T^+ R^- mutations, and 6 other constitutive mutations. For isolation method 3, there were 20 *nadI* T^- R^- mutations, 6 *nadI* T^+ R^- mutations, and 31 *nadI* T^- R^+ mutations.

diagram describing the linkage of markers in this region and a proposal for the arrangement of the *nadI* mutations is presented in Fig. 4.

Some *nadI* mutations excrete nicotinic acid. In the course of characterizing *nadI* mutants, it was noted that many of these strains excrete a compound that can feed *nadA* and *nadB* auxotrophs. In an attempt to characterize this phenomenon, a series of *nadI* mutants was checked for their ability to feed a series of multiple mutant strains that differed in their ability to use various pyridine compounds. The results (Table 8) indicate that strains constitutive for *nadA* and *nadB* excrete nicotinic acid. It may be that these strains overproduce NaMN, which breaks down to release nicotinic acid. Alternatively, excess NAD may break down to release nicotinamide, which is accumulated as nicotinic acid after deamination by the *pncA* function. Either of these events could be fortuitous or could represent a fail-safe device that prevents formation of excess pyridine nucleotides. Mutants defective only in NMN transport showed no feeding. The control strains showed that *pncB* mutants probably also excrete nicotinic acid and *pncA* mutants excrete nicotinamide. This is consistent with their position in the pyridine nucleotide cycle (Fig. 1).

DISCUSSION

The *nadB* region appears to encode a single cistron that is derepressed in response to pyridine starvation. The earlier suggestion that a positive control protein might be encoded at the *nadB* locus was based on the observation that *nadB* mutants appear to fall into two classes based on whether their extracts show quinolinate synthetase activity when mixed with extracts of a *nadA* mutant (30, 31). The *nadB* mutants lacking *nadA* activity were attributed to defects in a positive regulatory element (*nadR*) encoded at *nadB*. Three lines of evidence are presented here that argue against this possibility. (i) Mutants resistant to 6AN (which were the best candidates for mutants with regulatory defects) appear to have structural alterations of the first enzyme and are resistant to feedback inhibition; some of these mutants are temperature-sensitive auxotrophs that map within the *nadB* gene. These mutations do not appear to affect a separate regulatory protein. (ii) The *nadB* region appears to include only one complementation group. (iii) No *nadB* mutation tested, including large deletions, had a substantial effect on *nadA* gene transcription. While our findings argue against the existence of a positive regulatory element (*nadR*) encoded at the *nadB* locus, they do not suggest an alternative explanation for the data presented by Tritz (30) and Tritz and Chandler (31). Other difficulties with the hypothetical *nadR* gene have been pointed out by Griffiths et al. (14).

To identify components of the actual regulatory mechanism, we isolated mutants which show constitutive expression of both *nadA* and *nadB* genes. This constitutive expression cannot, in itself, be taken as evidence that these

TABLE 8. Excretion by *nadI*(R^-) mutants

Strain being fed		Growth on[a]:				*nadI* mutants tested as feeders			Control strains tested as feeders		
Strain	Relevant genotype	N	Na	NMN	Qa	T^- R^-[b]	T^+ R^-[c]	T^- R^+[d]	*pncB*[e]	*pncA*[f]	LT2[g]
TT399	*nadB*	+	+	+	+	+	+	−	+	+	−
TR6509	*nadB pncA*	−	+	+	+	+	+	−	+	−	−
TR5988	*nadB pncA pnuA*	−	+	−	+	+	+	−	+	−	−
TR6450	*nadB pncB*	−	−	+	+	−	−	−	−	−	−
TR8786	*nadC*	+	+	+	−	+	+	−	+	+	−

[a] Abbreviations: N, nicotinamide; Na, nicotinic acid; NMN, nicotinamide mononucleotide; Qa, quinolinic acid.
[b] *nadI262*, *nadI242*::Tn*10*, *nadI241*::Tn*10*.
[c] *nadI278*.
[d] *pnuA11*.
[e] *pncB165*::Tn*10*.
[f] *pncA155*::Tn*10*.
[g] Wild type.

mutants have a defective regulatory mechanism. One can imagine many situations in which a mutation might cause a reduced level of end product of a pathway and thereby cause a physiological derepression of the biosynthetic genes. For example, impaired assimilation of nicotinamide or leakage of pyridine from the cell could reduce the level of NAD, leading indirectly to derepression. We think that one of the classes of constitutive mutations described here is due to a limitation in NAD synthesis caused by a partial defect in the *nadD* biosynthetic enzyme. Initially, it seemed likely that the *nadI* mutations described here might also be constitutive because of indirect effects; this seemed especially likely since the mutations appeared to lie in the same operon as a gene involved in NMN transport (*pnuA*). Recently, the finding of rare *nadI*^{s} (superrepressor) mutations (and their suppression by the presence of many copies of cloned *nadA* or *nadB* sequences) suggests that the *nadI* gene described here does, in fact, encode a regulatory protein, most likely a repressor, that acts on both *nadA* and *nadB* genes (N. Zhu, B. M. Olivera, and J. R. Roth, manuscript in preparation). The association of the *nadI* gene with *pnuA* opens several interesting possibilities. The repressor of synthesis may be induced coordinately with transport of an exogenous pyridine source. Alternatively, a single protein may function both as a repressor of *nadA* and *nadB* and as a component of the transport system for NMN.

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