Genetic Analysis of the Nuo Locus That Encodes the Proton Translocating NADH Dehydrogenase in Escherichia Coli

Holly Jennifer Falk-Krzesinski
Loyola University Chicago

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GENETIC ANALYSIS OF THE *nuo* LOCUS THAT ENCODES THE PROTON TRANSLOCATING NADH DEHYDROGENASE IN *Escherichia coli*

A DISSERTATION SUBMITTED TO
THE FACULTY OF THE GRADUATE SCHOOL
IN CANDIDACY FOR THE DEGREE OF
DOCTOR OF PHILOSOPHY

DEPARTMENT OF MICROBIOLOGY AND IMMUNOLOGY

BY

HOLLY JENNIFER FALK-KRZESINSKI

CHICAGO, ILLINOIS
JANUARY 1998
ACKNOWLEDGMENTS

Looking back at the past five and a half years, I realize the vast number of people whose guidance, support, encouragement, and advice I relied on to complete my doctoral work. I thank my parents, Ileen and Ken Falk, as well as the rest of my family and friends for their love and support. I am grateful to my colleagues Dr. Barry McNamara, Dr. Dolph Ellefson, Christine Beatty, Dr. Suman Kumari, and Dr. Birgit Prüß for listening and sharing their ideas. I also want to thank my colleague, Dr. Thorsten Friedrich, for his long-distance guidance and willingness to share reagents, ideas, data, and time. A special thanks to my friend and colleague, Jennifer Nelms, whose friendship, love, and sincere interest in my research mean the world to me.

I extend my sincerest gratitude to the members of my dissertation committee, Drs. Marion Hulett, Robert Gennis, David Hecht, and Adam Driks for their critical evaluations and guidance. I especially want to thank my advisor, Dr. Alan Wolfe for his guidance on a daily basis and his unceasing confidence in me.

Finally, I wish to thank my wonderful husband, Mitch Krzesinski, for his love, friendship, and unconditional support. His encouragement always provided me with the strength to get through even the toughest days.
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<tr>
<td>ArcA</td>
<td>aerobic respiration control protein A</td>
</tr>
<tr>
<td>ATPase</td>
<td>adenosine triphosphatase</td>
</tr>
<tr>
<td>bp</td>
<td>base pairs</td>
</tr>
<tr>
<td>BSA</td>
<td>bovine serum albumin</td>
</tr>
<tr>
<td>cAMP</td>
<td>cyclic adenosine monophosphate</td>
</tr>
<tr>
<td>Cm</td>
<td>chloramphenicol</td>
</tr>
<tr>
<td>CRP</td>
<td>cAMP receptor protein</td>
</tr>
<tr>
<td>CTR</td>
<td>C-terminal region of NuoG encoded by the 3' end of <em>nuoG</em></td>
</tr>
<tr>
<td>C-terminus</td>
<td>carboxy terminus</td>
</tr>
<tr>
<td>DMSO</td>
<td>dimethyl sulfoxide</td>
</tr>
<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
</tr>
<tr>
<td>DNase</td>
<td>deoxyribonuclease</td>
</tr>
<tr>
<td>EDTA</td>
<td>ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>EtBr</td>
<td>ethidium bromide</td>
</tr>
<tr>
<td>Fe-S</td>
<td>iron-sulfur centers</td>
</tr>
<tr>
<td>FAD</td>
<td>flavin adenine dinucleotide</td>
</tr>
<tr>
<td>FMN</td>
<td>flavin mononucleotide</td>
</tr>
<tr>
<td>FNR</td>
<td>fumarate and nitrate reduction/regulation protein</td>
</tr>
<tr>
<td>Symbol</td>
<td>Description</td>
</tr>
<tr>
<td>--------</td>
<td>-------------</td>
</tr>
<tr>
<td>$g$</td>
<td>gravitational force</td>
</tr>
<tr>
<td>$h$</td>
<td>hour</td>
</tr>
<tr>
<td>IHF</td>
<td>integration host factor</td>
</tr>
<tr>
<td>LB</td>
<td>Luria-Bertani broth</td>
</tr>
<tr>
<td>kb</td>
<td>kilobase</td>
</tr>
<tr>
<td>Km</td>
<td>kanamycin</td>
</tr>
<tr>
<td>min</td>
<td>minute</td>
</tr>
<tr>
<td>MOPS</td>
<td>morpholinopropanesulfonic acid sodium acetate EDTA buffer</td>
</tr>
<tr>
<td>NAD$^+$</td>
<td>nicotinamide adenine dinucleotide, oxidized</td>
</tr>
<tr>
<td>NADH</td>
<td>nicotinamide adenine dinucleotide, reduced</td>
</tr>
<tr>
<td>NarL</td>
<td>nitrate reductase regulatory protein L</td>
</tr>
<tr>
<td>NDF</td>
<td>NADH dehydrogenase fragment</td>
</tr>
<tr>
<td>NDH</td>
<td>NADH dehydrogenase type-II</td>
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<tr>
<td>N-terminus</td>
<td>amino terminus</td>
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<tr>
<td>OD</td>
<td>optical density</td>
</tr>
<tr>
<td>ON</td>
<td>overnight</td>
</tr>
<tr>
<td>ONP</td>
<td>$o$-nitrophenol</td>
</tr>
<tr>
<td>ONPG</td>
<td>$o$-nitrophenyl-β-D-galactoside</td>
</tr>
<tr>
<td>ORF</td>
<td>open reading frame</td>
</tr>
<tr>
<td>PAGE</td>
<td>polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate buffered saline</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>PCR</td>
<td>polymerase chain reaction</td>
</tr>
<tr>
<td>RBS</td>
<td>ribosome binding site</td>
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<td>RNA</td>
<td>ribonucleic acid</td>
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<tr>
<td>RNase</td>
<td>ribonuclease</td>
</tr>
<tr>
<td>rRNA</td>
<td>ribosomal RNA</td>
</tr>
<tr>
<td>RT</td>
<td>room temperature</td>
</tr>
<tr>
<td>s</td>
<td>second</td>
</tr>
<tr>
<td>SDS</td>
<td>sodium dodecyl sulfate</td>
</tr>
<tr>
<td>SEM</td>
<td>standard error of the mean</td>
</tr>
<tr>
<td>SET</td>
<td>salt-EDTA-Tris buffer</td>
</tr>
<tr>
<td>sp act</td>
<td>specific activity</td>
</tr>
<tr>
<td>SSC</td>
<td>saline sodium citrate buffer</td>
</tr>
<tr>
<td>TAE</td>
<td>Tris-acetate-EDTA buffer</td>
</tr>
<tr>
<td>TB</td>
<td>tryptone broth</td>
</tr>
<tr>
<td>Tc</td>
<td>tetracycline</td>
</tr>
<tr>
<td>TCA</td>
<td>tricarboxylic acid</td>
</tr>
<tr>
<td>TMAO</td>
<td>triethylamine N-oxyde</td>
</tr>
<tr>
<td>Tris</td>
<td>tris(hydroxymethyl)aminomethane</td>
</tr>
<tr>
<td>vol</td>
<td>volume</td>
</tr>
<tr>
<td>wt</td>
<td>weight</td>
</tr>
<tr>
<td>X-gal</td>
<td>5-bromo-4-chloro-3-indolyl-β-D-galacto-pyranoside</td>
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</table>
ABSTRACT

Complex I (E.C. 1.6.99.3) of the bacterium *Escherichia coli* is considered to be the minimal form of the type-I NADH dehydrogenase, the first enzyme complex in the respiratory chain. Because of its small size and relative simplicity, the *E. coli* enzyme has become a model in the endeavor to identify and characterize the mechanism(s) by which cells regulate the synthesis and assembly of this large respiratory complex.

To begin dissecting the processes by which *E. coli* cells regulate the expression of *nuo* and the assembly of complex I, we undertook a genetic analysis of the fourteen structural gene locus *nuo* that encodes the fourteen Nuo subunits comprising *E. coli* complex I. We performed studies on an coisogenic collection of *nuo* mutants that focus on the physiological, biochemical, and molecular consequences caused by the lack of or defects in several Nuo subunits. Our findings provide insight into the regulation of *nuo* expression and complex I assembly. In particular, we acquired evidence that the peripheral subunit, NuoG, is essential for complex I function and that it plays a role in the regulation of *nuo* expression and/or the assembly of complex I. Furthermore, we demonstrated that Nuo subunits within the membrane arm of complex I may have dual functions, working in conjunction with NuoG to coordinate transcription at the *nuo* promoter thus providing a feedback mechanism of regulation. And, finally, we
uncovered a seemingly complicated scheme of multiple sigma factor interaction at the
nuo promoter.
CHAPTER I

INTRODUCTION

Regulation of Synthesis and Assembly of Multisubunit Complexes

The ability of a prokaryotic cell to tightly coordinate the synthesis and assembly of large multisubunit complexes is crucial to its ability to react in response to its environment (both internal and external) and to maintain efficient energy utilization. Cells have devised a number of mechanisms to accomplish such coordinated regulation. Three multisubunit complexes whose regulation is well understood are the *Escherichia coli* and *Salmonella typhimurium* flagellar apparatus (59, 70, 71), the *Rhodobacter capsulatus* photosystem components (7), and ribosomes (84).

Flagellar gene expression is coupled through a system of hierarchial operons in which the flagellar genes are clustered. The expression of one operon is dependent upon the product(s) produced by another through an alternative sigma factor and other transcription factors (71). Negative regulation of the late operons is achieved in part through the anti-sigma factor FlgM. FlgM binds to the product of the *fliA* gene, σ²₈, thus preventing expression of σ²₈-dependent operons (70, 71). However, when assembly of the flagellar apparatus reaches the stage of morphogenesis at which flagellin proteins can be exported from the cell (to form the flagellar filament) through the apparatus, FlgM is also exported out of the cell into the medium thus lowering its intracellular concentration.
In turn, $\sigma^{28}$ is released and becomes free to function in transcription initiation (59, 70). This mechanism serves as a "developmental checkpoint" that links the regulation of gene expression and assembly.

Expression of the *R. capsulatus* photosystem components are also tightly regulated (7). Expression of pigment biosynthesis genes is coupled to expression of the structural genes through various operons that overlap one another, designated "superoperons". This provides for a balanced synthesis of the light-harvesting and photopigment components. Furthermore, the various operons are controlled by different transcription factors including an aerobic repressor, a two-component regulatory system, and possibly an unidentified alternative sigma factor.

Many of the mechanisms that regulate ribosome synthesis and assembly are in the form of translation-dependent feedback inhibition (84). Certain free r-proteins can act as feedback inhibitors of translation of their own mRNA thereby coupling the synthesis of r-proteins with ribosome assembly. Other r-proteins can regulate the synthesis of multiple r-proteins from a polycistronic mRNA molecule.

Complex I in *E. coli* is also a large, multisubunit complex whose synthesis and assembly we hypothesize are likely to be regulated, perhaps by the very subunits that comprise complex I. Like many other multisubunit complexes, the genes that encode for complex I are clustered (38, 68). Since the genes that encode the subunits of complex I are not organized into several operons but rather localized within a single locus (*nuo*), we may focus on the concomitant regulation of the promoter proximal and promoter distal genes rather than looking for a hierarchical scheme of regulation. In thinking about this
possibility of regulation, we need to keep in mind that all of the subunits encoded by the 

nuo genes are known to have structural and/or enzymatic functions. The stoichiometry 
of "1" for each subunit of complex I (38, 68) suggests the presence of regulatory 
components which coordinately balance the synthesis of every subunit. Further evidence 
for a regulatory scheme is that the nuo locus is expressed in response to a number of 
environmental stimuli (12, 106).

E. coli complex I as a Model System

Researchers have begun to utilize complex I of E. coli, and that of its close 
relative S. typhimurium, to identify and characterize the mechanism(s) by which cells i) 
regulate the synthesis and assembly of this large respiratory complex (3, 12, 106), ii) to 
investigate the diverse physiological consequences caused by defects in this enzyme (4, 
16, 91, 126), and iii) to study the mechanism of electron and proton translocation (37, 
110, 114). These studies provide insight into bacterial energy metabolism and 
macromolecular complex synthesis and assembly. Moreover, because of its relatively 
small size and simplicity, E. coli complex I provides an excellent model system to study 
mitochondrial complex I. Ultimately, these efforts will contribute to the medical 
community’s understanding of the relationship between defects in complex I and the 
processes that lead to aging and diseases such as Parkinson’s disease.

To begin dissecting the processes by which E. coli cells regulate the expression of 
nuo and the assembly of complex I, we undertook a genetic analysis of the nuo locus. 
Here, we present the results of studies, performed on an isogenic collection of nuo
mutants, that focus on the physiological, biochemical, and molecular consequences caused by the lack of or defects in several Nuo subunits. In particular, we present evidence that NuoG, a peripheral subunit, is essential for complex I function and that it plays a role in the regulation of *nuo* expression and/or the assembly of complex I. We also present data consistent with a role for another subunit, the membrane subunit NuoA (a membrane subunit), in the regulation of *nuo* expression. Finally, we provide data that suggests multiple levels of sigma factor regulation at the *nuo* promoter.

**Complex I, The Enzyme**

Complex I (E.C. 1.6.99.3; NADH:ubiquinone oxidoreductase), a type-I NADH dehydrogenase that couples the oxidation of NADH-reducing equivalents to the generation of a proton motive force, is the first enzyme complex of the respiratory chain (2, 82, 109, 111, 116) (Fig. 1). It performs the reaction

\[
\text{NADH} + Q + 5H^+_n \rightarrow \text{NAD}^+ + \text{QH}_2 + 4H^+_p
\]

where Q refers to ubiquinone and \(H^+_n\) and \(H^+_p\) refer to the protons taken up from the negative side of the membrane and delivered to the positive side of the membrane, respectively (40). Complex I and related proton-pumping NADH dehydrogenases have been identified in a variety of eukaryotic organisms (111, 116 for review) as well as in the \(\alpha\)-, \(\beta\)-, \(\gamma\)-, and \(\delta\)-subdivisions of eubacteria (64, 66, 114, 119).
Fig. 1. Schematic of the *E. coli* aerobic respiratory chain. NADH reducing equivalents from glycolysis and the TCA cycle enter the respiratory chain through complex I, a type I NADH-dehydrogenase. Complex I couples the oxidation of NADH to the generation of a proton motive force. It passes the electrons from the oxidation step to the quinone pool where they then are passed on to either of two terminal oxidases and finally to oxygen. The proton motive force generated by complex I drives the formation of ATP. NDH-II, a type II NADH-dehydrogenase also oxidizes NADH, but does not generate a proton motive force.
**Escherichia coli NADH Dehydrogenases**

The *Escherichia coli* complex I, considered the minimal form of the enzyme, consists of fourteen polypeptide subunits (38, 67, 68, 73, 114), instead of the 40-50 structural and accessory subunits associated with the homologous eukaryotic mitochondrial enzyme (72, 111, 115, 116), and contains a numbers of prosthetic groups (FMN and Fe-S centers) (38, 67, 68). The *E. coli* enzyme is encoded by the fourteen gene locus, *nuo* (NADH:ubiquinone oxidoreductase), located between 49.2 and 49.6 min on the chromosome (16, 68). In addition, *E. coli* possesses a second, single polypeptide NADH dehydrogenase (type-II), NDH-II, which also oxidizes NADH and contains a FAD prosthetic group (61, 62). However, this process is not coupled to the generation of a proton motive force (73). This second NADH dehydrogenase is encoded by the *ndh* (NADH dehydrogenase) gene located at 22 min on the chromosome (16, 124).

**E. coli complex I and nuo Locus**

*E. coli* complex I resembles eukaryotic complex I in many ways (37, 38, 68, 114): it consists of subunits homologous to those found in all proton-translocating NADH:ubiquinone oxidoreductases (complex I) studied thus far (Table 1); it is comprised of a large number of subunits relative to the number that comprise other respiratory enzymes; it contains FMN and Fe-S center prosthetic groups; it performs the enzymatic reaction that does eukaryotic complex I; and it is sensitive to the a number of the same inhibitors. Additionally, it possesses an L-shaped topography (33, 46) like its *Neurospora crassa* homolog (58) and can be purified as three distinct fragments.
Table 1. The Nuo subunits and their homologs. *E.c. refers to *Escherichia coli, S.t. to *Salmonella typhimurium, P.c. to *Paracoccus denitrificans, T.t. to *Thermus thermophilus, B.t. to *Bov taurus, and N.c. to *Neurospora crassa. IP refers to iron-sulfur protein fraction, FP to flavoprotein fraction. *, mitochondrial-encoded. The data for various proteins comes from the following sources: *E. coli* and *B. taurus* proteins (114); *S. typhimurium* proteins (3 and Genbank accession numbers L22504 and L42521); *P. denitrificans* (120); *T. thermophilus* (122); *R. capsulatus* (26-28); *N. crassa* (5, 33). The predicted functions are based on data from a number of sources (37-40, 68).
<table>
<thead>
<tr>
<th>E. c.</th>
<th>S. t.</th>
<th>P. d.</th>
<th>T. t.</th>
<th>R. c.</th>
<th>B. t.</th>
<th>N. c.</th>
<th>Predicted function or cofactor binding</th>
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<tr>
<td>NuoA</td>
<td>NuoA</td>
<td>NQO7</td>
<td>NQO7</td>
<td>NuoA</td>
<td>ND3*</td>
<td>ND3*</td>
<td>1 X [4Fe-4S]</td>
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<tr>
<td>NuoB</td>
<td>NuoB</td>
<td>NQO6</td>
<td>NQO6</td>
<td>NuoB</td>
<td>PSST</td>
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<td>NuoC</td>
<td>NQO5</td>
<td>NQO5</td>
<td>NuoC</td>
<td>30 IP</td>
<td>Nuo31</td>
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<td>NuoD</td>
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<td>NQO4</td>
<td>NuoD</td>
<td>49 IP</td>
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<td>NQO2</td>
<td>NQO2</td>
<td>NuoE</td>
<td>24 FP</td>
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<td>NQO1</td>
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<td>51 FP</td>
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<td>NADH-binding; FMN; 1 X [4Fe-4S]</td>
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<td>NuoH (Ndha)</td>
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<td>ND1*</td>
<td>ubiquinone-binding</td>
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<td>NuoI</td>
<td>NuoI</td>
<td>NQO9</td>
<td>NQO9</td>
<td>NuoI (Ndhi)</td>
<td>TYKY</td>
<td></td>
<td>2 X [4Fe-4S]</td>
</tr>
<tr>
<td>NuoJ</td>
<td>NuoJ</td>
<td>NQO10</td>
<td>NQO10</td>
<td>NuoJ</td>
<td>ND6*</td>
<td>ND6*</td>
<td></td>
</tr>
<tr>
<td>NuoK</td>
<td>NuoK</td>
<td>NQO11</td>
<td>NQO11</td>
<td>NuoK</td>
<td>ND4L*</td>
<td>ND4L*</td>
<td></td>
</tr>
<tr>
<td>NuoL</td>
<td>NuoL</td>
<td>NQO12</td>
<td>NQO12</td>
<td>NuoL</td>
<td>ND5*</td>
<td>ND5*</td>
<td>proton translocation</td>
</tr>
<tr>
<td>NuoM</td>
<td>NuoM</td>
<td>NQO13</td>
<td>NQO13</td>
<td>NuoM</td>
<td>ND4*</td>
<td>ND4*</td>
<td>proton translocation</td>
</tr>
<tr>
<td>NuoN</td>
<td>NuoN</td>
<td>NQO14</td>
<td>NQO14</td>
<td>NuoN</td>
<td>ND2*</td>
<td>ND2*</td>
<td>proton translocation</td>
</tr>
</tbody>
</table>
Whereas eukaryotic complex I can be dissected into a peripheral arm and a membrane arm, the *E. coli* enzyme is comprised of three subcomplexes referred to as the peripheral, connecting, and membrane fragments (67) (Fig. 2A).

The organization of *nuo* (38, 68, 114) (Fig. 2B) and related genes is conserved in several other bacteria, including *Salmonella typhimurium* (3), *Paracoccus denitrificans* (118), *Rhodobacter capsulatus* (27), and *Thermus thermophilus* (122). The 5' half of the *nuo* locus contains a promoter (*nuoP*) located upstream of *nuoA* (3, 12, 114) and the majority of genes that encode subunits homologous to the nuclear-encoded subunits of eukaryotic complex I (38, 67, 68, 114). In contrast, the 3' half contains the majority of the genes that encode subunits homologous to the mitochondrial-encoded subunits of eukaryotic complex I (38, 67, 68, 114), as well as sequences resembling transcriptional termination signals (114).

The subunit composition of the three fragments of *E. coli* complex I correlates approximately with the organization of the fourteen structural genes, *nuoA*-*nuoN* (38, 68, 114) (Fig. 2A & B). Whereas the nuclear homologs NuoE, NuoF, and NuoG constitute the peripheral fragment (also referred to as the NADH dehydrogenase fragment or NDF), the nuclear homologs NuoB, NuoC, NuoD, and NuoI comprise the connecting fragment. The mitochondrial homologs NuoA, NuoH, NuoJ, NuoK, NuoL, NuoM, and NuoN constitute the membrane fragment (67). Based on predictions from conserved sequence motifs (38, 63, 68), complex I inhibitor studies (37), and evolutionary relationships (39, 40), the *E. coli* complex I subunits which bind substrates, harbor redox groups, and participate in proton translocation have been identified putatively (Table 1). The
peripheral fragment is believed to be the electron input site (38); the membrane fragment
contains the ubiquinone-binding site (38, 68) and is thought to be involved in proton
translocation (39, 40, 63).

**Similarity of *E. coli* complex I subunits to other bacterial enzymes**

The subunits of complex I resemble subunits found in other bacterial electron
transfer and proton transporting enzymes (30, 39, 40, 111). The electron transfer moiety
of complex I is related to three different bacterial enzymes. The first enzyme is a soluble
NAD⁺-reducing hydrogenase encoded by the *hox* locus in the chemolithotrophic
bacterium *Alcaligenes eutrophus* (90, 107). This enzyme is composed of four subunits: α
(encoded by *hoxF*), β (encoded by *hoxH*), γ (encoded by *hoxU*), and δ (encoded by
*hoxY*). Whereas the β/δ dimer is splits H₂ (H₂ → 2H⁺), the γ/δ dimer functions as an
NADH oxidoreductase (diaphorase; NAD⁺ → NADH). The α/γ dimer also contains an
FMN prosthetic group and a number of Fe-S clusters. NuoE, NuoF, and NuoG share
some homology to the α and γ subunits whereas NuoB and NuoD share homology with
the δ and β subunits (Table 2). The gene order of the *hox* locus is conserved with respect
to the corresponding *nuo* genes. The second enzyme is the hydrogenase 3 of *E. coli*
formate hydrogenlyase encoded by the *hyc* operon (11). This enzyme couples the
oxidation of formate (formate → CO₂) to proton reduction (2H⁺ → H₂). All of the Hyc
subunits share homology with Nuo subunits: HycE is related to NuoC and NuoD; HycG
is related to NuoB, which also contains an Fe-S center; HycF, a ferrodoxin, is related to
NuoI, also possessing Fe-S centers; and the membrane proteins HycC and HycD,
Fig. 2. Schematic of the *E. coli* complex I and the *nuo* locus. Adapted with permission from T. Friedrich (38, 67, 68, 114). (A) *E. coli* complex I is comprised of three distinct fragments: the peripheral (light gray), connecting (white), and membrane (dark gray) fragments. The peripheral fragment (NDF) is comprised of the nuclear homologs NuoE, F, and G and exhibits NADH dehydrogenase activity that oxidizes NADH to NAD$^+$; the connecting fragment is comprised of the nuclear homologs NuoB, C, D, and I; and the membrane fragment is comprised of the mitochondrial homologs NuoA, H, and J-N and catalyzes ubiquinone (Q) to its reduced form (QH$_2$). (B) The *E. coli nuo* locus encodes the fourteen Nuo subunits that constitute complex I. The 5' half of the locus contains a previously identified promoter (*nuoP*) and the majority of genes that encode the peripheral and connecting subunits (light gray and white, respectively). The 3' half of the locus contains the majority of the genes encoding the membrane subunits (dark gray). The 3' end of *nuoG* encodes a C-terminal region (CTR) of the NuoG subunit (hatched).
A peripheral connecting membrane

NADH

NAD^+

3 FeS

Q

QH_2

cytoplasm

periplasm

B

CTR

~15,700 bp
Table 2. *E. coli* complex I subunits and their homologs in other bacterial electron transfer enzymes and transporters. N-term., N-terminus; C-term., C-terminus; PTS, phosphotransferase systems. *A. eutrophus, Alcaligenes eutrophus; R. meliloti, Rhizobium meliloti.* This table is reproduced with permission from T. Friedrich; references for each relationship have been reviewed (30, 39, 40, 111).
<table>
<thead>
<tr>
<th>E. coli complex I</th>
<th>A. eutrophus NAD^+ -reducing hydrogenase</th>
<th>E. coli formate hydrogenase</th>
<th>Bacterial hydrogenases</th>
<th>Bacterial glucose dehydrogenases</th>
<th>R. meliloti K^+ / H^+ antiporter</th>
<th>Bacillus C-125 Na^+ / H^+ antiporter</th>
<th>Bacterial PTS</th>
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<tbody>
<tr>
<td>NuoE</td>
<td>α N-term. (HoxF)</td>
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<td></td>
<td></td>
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<tr>
<td>NuoF</td>
<td>α C-term. (HoxF)</td>
<td></td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>NuoG N-term.</td>
<td>γ (HoxU)</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<td></td>
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<tr>
<td>NuoB δ (HoxY)</td>
<td>HycG</td>
<td>small subunit</td>
<td></td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>NuoC</td>
<td>HycE N-term.</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>NuoD β (HoxH)</td>
<td>HycE C-term.</td>
<td>large subunit</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>NuoH</td>
<td>HycD</td>
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<td></td>
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<td></td>
</tr>
<tr>
<td>NuoI</td>
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<td>NuoL</td>
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<td>NuoM</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>PhaA</td>
<td>ORF1</td>
<td>sugar permease</td>
</tr>
</tbody>
</table>

PTS: Phosphotransferase System
thought to be involved in ubiquinone binding, are similar to NuoL and NuoH, respectively (Table 2). In contrast to the hox genes, the hyc genes do not correspond to the order of the nuo genes.

The third enzyme is bacterial glucose dehydrogenase (glucose:ubiquinone oxidoreductases) (74). This family of enzymes catalyzes the oxidation of glucose to gluconolactone while reducing ubiquinone (Q→QH₂). They can be inhibited by a number of compounds that also inhibit complex I by interfering with its interaction with ubiquinone (36, 37). The N-terminus of each of these enzymes is homologous to NuoH (Table 2).

The proton translocating moiety of complex I is related to at least two bacterial enzymes, i.e. certain antiporters and phosphoenolpyruvate-dependent phosphotransferase systems (PTS). The membrane intrinsic PhaA and PhaD subunits of the Rhizobium melioti K⁺/H⁺ antiporter share homology with NuoL and NuoM, respectively; likewise, a related Na⁺/H⁺ antiporter in Bacillus C-125 contains a protein encoded ORFl that shares homology with NuoL (Table 2). The pore-forming sugar permeases of the bacterial PTS (93) also display some homology to NuoL as well as NuoM (Table 2).

**Evolution of E. coli complex I**

A possible mechanism of evolution of large multisubunit assemblies is that the assembly arose by the accretion of its various component activities. This type of modular evolution has been described for the bacteriophage lambda and for ATP synthases (18, 92, 111-113). For the bacteriophage lambda, the genes that encode the head are clustered
together and those that encode the tail are clustered together. It has been proposed that lambda phages exchange these gene clusters and so evolution is thought to operate at the level of the individual functional units, not at the level of the intact virus (18, 92). The ATP synthases are comprised of two membrane-bound structural modules, the extrinsic $F_1$ (the ATPase module) and the intrinsic $F_0$ (the proton channel module). The clustering of the genes that encode for each module and a conservation of gene order in various organisms support modular evolution for this enzyme complex as well (112, 113).

The $nuo$ genes are also clustered and their arrangement is conserved (refer to the "E. coli complex I and $nuo$ Locus" section above). And, E. coli complex I is similar to a number of other bacterial enzymes. These pieces of data are consistent with the possibility that E. coli complex I has evolved by fusion of preexisting modules for electron transfer and proton translocation (30, 35, 39, 40, 111, 116).

The E. coli complex I is believed to have evolved first from a hydrogenase (NuoB, NuoD), a ferredoxin (NuoI), a transport protein (NuoM), a protein possessing a quinone reduction site (NuoH), and a protein of unknown function (NuoC). A triplication event of NuoM created NuoN and NuoL within the complex. The enzyme that resulted was a common ancestor of complex I and formate hydrogenlyase. It likely used $H_2$ as an electron donor working through NuoB-D and NuoI, while NuoL-N functioned in proton translocation, and NuoH served as the ubiquinone-binding site. A more sophisticated $H_2$::quinone oxidoreductase evolved upon the acquisition of NuoA, NuoJ, and NuoK (whose functions are unknown). Later in evolution, this primitive enzyme complex
acquired the NADH dehydrogenase component (NuoE, NuoF, NuoG) which resulted in complex I, an NADH:ubiquinone oxidoreductase.

Assembly of complex I

In the fungus Neurospora crassa, cells assemble complete complex I en bloc from two independently assembled subcomplexes. Once both subcomplexes (arms) are formed, they assemble into a single enzyme complex at the mitochondrial matrix (31, 34, 81, 98, 108), presumably through the actions of accessory proteins (111). The peripheral arm, comprised of only nuclear-encoded subunits, assembles independently of the membrane arm in cells grown in the presence of chloramphenicol, an antibiotic that inhibits mitochondrial protein synthesis (34). The membrane arm, comprised of both nuclear- and mitochondrial-encoded subunits, also assembles independently by cells grown under Mn^{2+}-limiting conditions, a condition that prevents the synthesis of the peripheral arm (98). Accumulation of either arm occurs even if a subunit from one arm is genetically disrupted (25, 31, 41, 54, 57, 81, 100).

The similarity of complex I in E. coli to the N. crassa enzyme in terms of evolution and subunit functionality suggests that the E. coli and N. crassa enzymes may also share similar modes of assembly (38-40, 68). The organization of the nuo locus is consistent with the hypothesis that E. coli complex I assembly also proceeds first by constructing independently assembled subcomplexes (38, 68). Perhaps, throughout the evolution of complex I, regulatory components of each protein assembly remained within the nuo locus to coordinate regulation of its two halves, ensuring proper assembly of a
complete and functional complex I. Since *E. coli* harbors all of its *nuo* components in a single cellular compartment, the regulation of expression and assembly would, however, differ between *E. coli* and eukaryotes.

**Regulation of *nuo* by External Regulators**

Many components of the respiratory chain are known to be extensively regulated at the level of transcription. In response to various electron donors and electron acceptors, global transcriptional regulators (FNR, NarL, and ArcA) coordinately regulate the expression of the dehydrogenases and terminal reductases of the respiratory chains (48, 60, 109).

An extensive examination of the effect of external regulators on *nuo* transcription in *E. coli* was performed by Bongaerts *et al.* (12) and Tran *et al.* (106). The first study examined the role of electron acceptors, electron donors, and global transcriptional regulators on *nuo* transcriptional control, while the second study focused on *nuo* expression during formate respiration. These researchers determined that the electron acceptors O\(_2\) and nitrate induce *nuo* transcription via the regulators ArcA, NarL, FNR, and IHF. In contrast, cAMP-CRP seemed not to be involved. ArcA acted as a strong repressor during anoxic conditions. NarL acted as a strong activator under the anoxic conditions in the presence of nitrate. The global regulators FNR and IHF behaved as weak repressors, exerting little effect on *nuo* transcription under anaerobic conditions. This pattern of expression differs from that of *ndh*; *ndh* is primarily regulated by FNR (44). Additionally, the electron acceptor fumarate (via an unknown mechanism) served
to increase *nuo* expression anaerobically. In terms of electron donors, *nuo* transcription was increased aerobically by C$_4$ dicarboxylates (*e.g.* succinate, fumarate, and malate) and acetate. Growth on substrates supplying high levels of NADH, *e.g.* glucose or glycerol, however, did not stimulate *nuo* expression either aerobically or anaerobically. Furthermore, a comparison of operon and protein fusions demonstrated that the expression of *nuo* is regulated at the level of transcription, not translation, by electron donors and acceptors.

It is likely that *E. coli* possesses both its NADH dehydrogenases to effectively maintain a redox balance in the cell (12, 16, 35, 52, 106) and to maximize energy production/conservation (12, 16). *nuo* is expressed in the presence of many electron donors and acceptors under both aerobic and anoxic conditions (12, 106), whereas *ndh* is expressed primarily under aerobic conditions (44, 104). Both are repressed during fermentation, when NADH cannot be reoxidized (12). It has been proposed that cells may regulate the NADH/NAD$^+$ levels in the cell and the amount of energy recovered from NADH oxidation by controlling the relative levels and/or activities of the two NADH dehydrogenases (16). Additionally, the presence of multiple NADH dehydrogenases in *E. coli*, as well as other dehydrogenases, allows the cell to modulate the electron flow through its respiratory chains in response to its environment (106, 109).

**nuo promoter elements**

Putative σ$^{70}$-dependent -35/-10 sites were identified within the *nuo* promoter region for *E. coli* (12, 114) and a -10 site was described in *S. typhimurium* (3). However,
these sites were described as poor consensus sequences. Furthermore, in the study performed by Bongaerts et al. (12), primer extension experiments revealed two transcriptional start sites: one close to the start site described previously by Weidner et al. (114) and a more distal site located at -173/-172. Additionally, two distinct regulatory regions were defined. A proximal promoter region was shown to be required for regulation by O_2 and nitrate and the global regulators ArcA, NarL, and FNR, whereas a distal promoter region was shown to be required for transcriptional stimulation by C_4 carboxylates and acetate. These findings suggest that the *nuo* operon may possess more than one promoter upstream of *nuoA*.

**Defects in complex I**

Defects in bacterial complex I cause pleiotropic phenotypes. In *E. coli* and/or *S. typhimurium*, the defects affect the ability of cells to perform chemotaxis (91), to grow on certain carbon sources (4, 8, 16, 91, 124), to survive stationary phase (126), to perform energy-dependent proteolysis (4), to regulate the expression of the *dnaN* gene (75), and maintain virulence (6). In *Rhodobacter capsulatus*, the defects prevent cells from growing photosynthetically under anaerobiosis (28); while in *Myxococcus xanthus*, the defects cause cells to halt the production of extracellular proteins and to fail to develop and sporulate properly (66).

Defects in eukaryotic complex I also elicit severe physiological consequences. In the fungi *Neurospora crassa* and *Aspergillus niger*, defects in complex I have been shown to cause a number of defects, including a reduction in growth rate (1, 31, 54, 81,
115) and poor conidiation (54). In humans, complex I defects are associated with idiopathic Parkinson's disease, Leber's Hereditary Optic Neuropathy, and other severe neuromuscular degenerative diseases (9, 13, 96, 99). These diseases predominately affect brain and muscle, organs with the highest demand for ATP (116). Furthermore, complex I deficiency has also been shown to play an important role in the aging process (21, 86, 89) and perhaps even in drug addiction (22). Since eukaryotic mitochondrial complex I consists of 40-50 subunits, its size and complexity make it the least understood proton translocating complex. This lack of understanding hampers efforts to define the role that defects in complex I play in human disease.
CHAPTER II
MATERIALS AND EXPERIMENTAL METHODS

Chemical and Biological Reagents

Restriction enzymes were purchased from Promega Corporation (Madison, WI), Gibco-BRL Life Technologies (Gaithersburg, MD), or New England BioLabs (Beverly, MA). Antibiotics were purchased from Sigma Chemical Company (St. Louis, MO). Enzymes and substrates were obtained from Boehringer Mannheim (Indianapolis, IN) or Sigma Chemical Company. Radiolabeled materials were purchased from Amersham (Arlington Heights, IL) and the bicinchoninic acid (BCA) protein assay reagent was obtained from Pierce Biochemicals (Rockford, IL).

Bacterial Strains and Mutant Alleles

All strains are derivatives of *E. coli* K-12 and are listed in Table 3. The Δ*nuoG1* and *nuoG2* mutant alleles were constructed by first subcloning a 7.0-kb EcoRI-PstI *nuo* fragment from pAJW105ΔPstI, a plasmid that encompasses the 3' end of *nuoF* to the 3' end of *nuoL* (refer to Fig. 5), into the site-directed mutagenesis vector pALTER (Promega Corporation, Madison, WI). Then, following the manufacturer’s instructions, two rounds of site-directed mutagenesis were performed using the primers CTR1 (5'-GTTTGGCAGCGATGAGTCGACACAGCGTGCTCCGG-3') and CTR2
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<td><em>polA</em>(Ts) <em>thi-1 thr</em>(am)-1 *leuB6 <em>hisF</em>-4 <em>rpsL136</em> *lacY1 xyl-5 <em>ara14</em></td>
<td>(50, 87)</td>
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<tr>
<td></td>
<td><em>tonA</em>31 <em>tsx</em>-78 <em>rha</em> <em>zig::Tnl</em></td>
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<td>CP875</td>
<td><em>thi-1 thr</em>(am)-1 *leuB6 <em>hisF</em>-4 <em>rpsL136</em> <em>lacY1 ΔlacX74</em> <em>lacY</em></td>
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<td>K-12</td>
<td>prototrophic wild-type</td>
<td>ATCC #23716</td>
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</tbody>
</table>

<p>| Nuo mutants: |                                                                                   |                     |
| AJW844       | CP875 <em>nuoB::Km</em>                                                                    | MCN021 X CP875→Km\textsuperscript{R} Nuo⁻ |
| AJW853       | CP875 <em>nuoB</em>-C::Cm\textsuperscript{b}                                                 | MWC230 X CP875→Cm\textsuperscript{R} Nuo⁻ |
| AJW851       | CP875 <em>nuoF::miniTnl0Cm\textsuperscript{c}                                           | ZK1363 X CP875→Cm\textsuperscript{R} Nuo⁻ |
| CP938        | CP875 Δ(<em>nuoF</em>-L)-1                                                                 | (91)                |
| CP910        | CP875 <em>nuoG::Tnl0</em>1\textsuperscript{a}                                               | (91)                |
| CP932        | CP366 <em>nuoG::Tnl0</em>1\textsuperscript{a}                                               | (91)                |
| AJW931       | CP366 Δ</em>nuoG1*\textsuperscript{d}                                                   | This study          |
| AJW1516      | CP875 Δ<em>nuoG1</em>\textsuperscript{d}                                                   | This study          |
| AJW1470      | CP366 <em>nuoG2</em>\textsuperscript{e}                                                    | This study          |
| AJW1517      | CP875 <em>nuoG2</em>\textsuperscript{e}                                                    | This study          |
| AJW845       | CP875 <em>nuoH::Km</em>                                                                     | ND1-Kan\textsuperscript{R} X CP875→Km\textsuperscript{R} Nuo⁻ |
| AJW846       | CP875 <em>nuol::Km</em>                                                                     | MCN091 X CP875→Km\textsuperscript{R} Nuo⁻ |
| AJW852       | CP875 *nuoM::miniTnl0Cm\textsuperscript{c}                                           | ZK1362 X CP875→Cm\textsuperscript{R} Nuo⁻ |
| AJW847       | CP875 <em>nuoN::Km</em>                                                                     | ANN141 X CP875→Km\textsuperscript{R} Nuo⁻ |
| AJW932       | CP366 pHFl 7r integrate                                                              | This study          |
| AJW1459      | AJW931 pAJW105ΔPstI\textsuperscript{g} integrate                                     | This study          |
| AJW1472      | AJW1470 pAJW105ΔPstI\textsuperscript{g} integrate                                     | This study          |</p>
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<td>(126)</td>
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<td>(10)</td>
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<td>ANN141</td>
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<td>(10)</td>
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**Sigma factor mutants:**

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<td>(123)</td>
</tr>
<tr>
<td>AJW1015</td>
<td>$\text{CP875} \text{rpoS(katF)}::\text{Km}; \text{RpoS(}\sigma_{58}^c\text{)}_{\text{Ts}}$</td>
<td>ZK1000 X CP875→Km$^R$</td>
</tr>
<tr>
<td>AJW1409</td>
<td>$\text{CP875} \text{rpoN}::\text{Tn10-1}^a; \text{RpoN(}\sigma_{54}^c\text{)}_{\text{Ts}}$</td>
<td>YMC18 X CP875→Tc$^R$</td>
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<tr>
<td>ZK1000</td>
<td>$\text{rpoS(katF)}::\text{Km}$</td>
<td>R. Kolter</td>
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</tbody>
</table>
Strain | Relevant genotype | Source or reference
---|---|---
YMC18 | rpoN::Tn10-1<sup>a</sup> | A. Ninfa

Others:

<table>
<thead>
<tr>
<th>Strain</th>
<th>Relevant genotype</th>
<th>Source or reference</th>
</tr>
</thead>
</table>
| AJW803 | CP875 ΔacsA::Km | S. Kumari

<sup>a</sup> nuoG::Tn10-1 and zig::Tn10 confer Tc<sup>R</sup>; rpsL136 confers Str<sup>R</sup>.

<sup>b</sup> The Cm cassette is located in the intergenic region between nuoB and nuoC.

<sup>c</sup> These mutations have been renamed to reflect their location within the nuo locus.

<sup>d</sup> The ΔnuoG1 mutation is a 235-bp deletion of a 3′ region of nuoG.

<sup>e</sup> The nuoG2 mutation is a 235-bp tandem duplication of a 3′ region of nuoG.

<sup>f</sup> pHF17 confers Ap<sup>R</sup> and carries the ΔnuoG1 allele.

<sup>g</sup> pAJW105ΔPsl confers Ap<sup>R</sup> and carries the wild-type nuoG allele.

(5′-GCGCGCATCTTGAGGTCGACAAGGAGGCACAACAA-3′), to create two unique SalI sites flanking the 3′ region of nuoG that encodes a C-terminal region (CTR) of the NuoG subunit. Next, these sites were used for cloning purposes to construct one plasmid (pHF17) that harbored a 235-bp deletion of the CTR (allele ΔnuoG1) and one plasmid (pHF18) that harbored a 235-bp tandem duplication of the CTR (allele nuoG2). HindIII fragments from pHF17 and pHF18 were subsequently subcloned into the temperature-sensitive suicide vector pMAK705 (52) to yield plasmids pHF68 and pHF69, respectively. Finally, alleles ΔnuoG1 and nuoG2 were introduced into the chromosome (Fig. 3) by means of homologous recombination following transformation with i) plasmids pHF17 and pHF18, respectively, into the Nuo<sup>+</sup> PolA<sup>Ts</sup> host strain CP366 (50, 87) or ii) plasmids pHF68 and pHF69, respectively, into the closely related Nuo<sup>+</sup> strain CP875 (52). Resultant recombinants were screened for the ΔnuoG1 deletion or the nuoG2 duplication by whole-cell PCR (94) using primers G1 (5′-
TTGGCGATCCGGGCGTGC-3') and H1 (5'-CAACCACCCAACCGGAC-3') that flank the nuoG CTR (Fig. 3). The wild-type nuoG allele produced a 717-bp product, the ΔnuoG1 allele produced a 482-bp product, and the nuoG2 allele produced a 952-bp product (Fig. 4). One CP366 ΔnuoG1 recombinant (designated strain AJW931), one CP366 nuoG2 recombinant (AJW1470), one CP875 ΔnuoG1 recombinant (AJW1516), and one CP875 nuoG2 recombinant (AJW1517) were selected for further study. The nuo mutant strains CP910 (allele nuoG::Tnl0-l) and CP938 (Δ(nuoF-L)-1) are derivatives of the nuo wild-type strain CP875, while CP932 (nuoG::Tnl10-1) is a derivative of the wild-type strain CP366 (91). To add to this isogenic set of nuo mutants, generalized transduction, using the phage P1kc (101), or transformation using chromosomal DNA was used to transfer mutant nuo alleles from a variety of genetic backgrounds (10, 43, 126) into strain CP875. The location of the nuoB::Km mutation (strain AJW844) relative to that of mutations nuoB-C::Cm and nuoF::miniTnl0Cm was verified genetically (97.4% and 70.8% linkage, respectively) and confirmed by Southern blot analysis (data not shown).

A strain carrying both the wild-type and ΔnuoG1 alleles on its chromosome was isolated following an integrative, homologous recombination event by the Campbell-type mechanism (88, 125) between pAJW105ΔPstI, which carries the wild-type nuoG allele, and the ΔnuoG1 polA(Ts) strain AJW931 (Fig. 5). Following transformation and a shift in temperature from 32°C to 42°C, a single, non-reciprocal, homologous recombination event between the cloned insert in pAJW105ΔPstI and the nuo locus in AJW931 resulted
Fig. 3. Wild-type \textit{nuoG} and mutant $\Delta \textit{nuoG}1$ and \textit{nuoG}2 alleles. $+1$, \textit{nuo} transcription initiation site (114). Restriction enzyme sites: E, \textit{EcoRI}; P, \textit{PstI}; S, \textit{SalI} derived by site-directed mutagenesis to facilitate construction of mutant alleles. CTR (hatched), a 235-bp 3' region of \textit{nuoG} present in the wild-type \textit{nuoG}, deleted in the $\Delta \textit{nuoG}1$ allele, and duplicated in tandem in the \textit{nuoG}2 allele. Inverted triangle, location of the CTR deletion. G1 and H1, flanking primers used to amplify CTR.
Fig. 4. Products of PCR amplification to confirm the mutant ΔnuoG1 and nuoG2 alleles. PCR was performed with plasmid or chromosomal DNA as described in Materials and Experimental Methods using primers G1 and H1 (see Fig. 3). The products were electrophoresed on a 1.8% agarose gel containing 10 µg/ml EtBr. Expected product sizes: wild-type nuoG allele, 717-bp; ΔnuoG1 allele, 482-bp; nuoG2 allele, 952-bp. Markings on the left indicate the sizes of the ΦX174 HaeIII DNA marker (Gibco-BRL; marker sizes: 1350-bp, 1080-bp, 870-bp, 600-bp, 310-bp). Lanes are as marked.

in a partial, non-tandem duplication of the nuo locus at either end of the vector sequence (50, 87). The resultant strain was designated AJW1459. A similar strain (AJW932) was constructed when the plasmid pHF17, which carries the mutant ΔnuoG1 allele, was integrated into the Nuo+ PolA Ts strain CP366 in the same manner. Likewise, a strain (AJW1472) carrying both the wild-type nuoG and mutant nuoG2 alleles on its chromosome was constructed when the plasmid pAJW105ΔPstI was integrated into the nuoG2 polA(Ts) strain AJW1470. Maintenance of integration was verified following each experiment by confirming the vector-encoded ampicillin resistance of each strain at 42°C (50) and the concurrent presence of both the wild-type nuoG and ΔnuoG1 or nuoG2 alleles within the chromosome by PCR analysis using primers G1 and H1.
Fig. 5. Construction of the cointegrate strain by integrational, homologous recombination. Vector-derived nuo sequence (white); chromosome-derived nuo sequence (gray); vector sequence (dotted line); ampicillin resistance, ApR. Other designations as in Fig. 3 legend. The plasmid pAJW105ΔPstI encompasses the 3' end of nuoF to the 3' end of nuoL and carries the wild-type nuoG allele. This plasmid was transformed into competent AJW931 (ΔnuoG1 polA(Ts)) cells. Cells in which the plasmid had integrated into the chromosome by homologous recombination were identified by selection for ampicillin resistance at the restrictive temperature, 42°C. Not shown: Strain AJW932 was constructed similarly, except that the plasmid pHF17, which carries the ΔnuoG1 allele, was integrated into the Nuo+ PolA Ts strain CP366. Strain AJW1472 was constructed in a similar manner by integrating pAJW105ΔPstI into strain AJW1470 (nuoG2 polA(Ts)). The concurrent presence of both the wild-type and mutant nuoG alleles in each of these strains was verified by PCR analysis with primers G1 and H1.
Integration at 42 °C
**Media and Growth Conditions**

Cells were grown with aeration in tryptone broth (TB), composed of 1% (wt/vol) tryptone and 0.5% (wt/vol) sodium chloride, or in Luria-Bertani media (LB), composed of TB and 0.5% (wt/vol) yeast extract (78). When necessary, 100 µg/ml ampicillin, 15 µg/ml tetracycline, 34 µg/ml chloramphenicol, or 40 µg/ml kanamycin was added. Cells were grown at 32°C, unless otherwise stated. To obtain growth curves, cells were grown in LB (supplemented with the appropriate antibiotics) to mid-exponential phase (OD$_{610}$ 0.35-0.4), then diluted $10^{-2}$ into fresh TB (without antibiotics), and aerated until they reached stationary phase. To obtain samples for β-galactosidase assays, cells were grown the same as for growth curves and samples were removed at the indicated time points. To test for the ability to use acetate as the sole carbon source, cells were grown in TB at 32°C to mid-exponential phase prior to harvesting and resuspension at $10^{-5}$ (vol of 100 µl). 50 µl of the diluted culture was spread on M63 minimal media plates (91, 101) supplemented with 25 mM sodium acetate (pH 7.0). Each plate was incubated at 32°C for 55.5 h. When whole cell lysates were required, cells were harvested by centrifugation at 3500 x g for 10 min at 4°C, washed once with phosphate-buffered saline (PBS), and lysed by sonication. 100 µl of the whole cell lysate was used to determine either the total protein concentration of each sample by the BCA reagent method of Smith et al. 1985, using bovine serum albumin (BSA) as a standard (103) (Pierce, Rockford, IL), or to perform β-galactosidase assays described below.
Swarm Assays

Cells were aerated in TB supplemented with the appropriate antibiotics at 32°C to mid-exponential phase (OD$_{610}$=0.35-0.4). Tryptone swarm plates were 0.25% agar in the same media. Antibiotics were not added. A 5 µl aliquot (10$^6$ to 10$^7$ cells) was inoculated at the center of the surface of each swarm plate. Swarm plates were incubated at 32°C in a humid environment until the outermost, serine ring reached the edge of the petri plate (117). The plates were then examined for the presence of the inner, aspartate ring (117). The absence of this inner ring was taken as indicative of the Nuo- phenotype, i.e. the lack of functional complex I (91).

Plasmid and Chromosomal DNA Preparation

Plasmid preparations were performed according to the alkaline lysis procedure (95), using either the Promega Wizard Miniprep Purification System (Madison, WI) or the QIAGEN plasmid midiprep kit (Santa Clarita, CA). Chromosomal DNA was prepared as described (83) based on the principle of salting-out proteins. Cells grown in 3 ml LB were harvested, washed 1X in PBS, resuspended in 1 ml SET buffer (75 mM NaCl, 25 mM EDTA, 20 mM Tris-HCl [pH 7.5]), and lysed in the presence of 1 mg/ml lysozyme. Preparations were incubated at 37°C for 0.5 h, and then incubated at 55°C for 1 h treated with 0.1 vol of 10% SDS and 1 mg/ml proteinase K. Cellular proteins were precipitated by the addition of 0.33 vol of 5 M NaCl and 1 vol chloroform followed by incubation for 30 min at RT on a rotating vertical wheel. The chromosomal DNA was precipitated from the aqueous phase by the addition of 1 vol isopropanol followed by
centrifugation at 3,200 x g for 5 min. The DNA was washed 1X in 70% ethanol, dried, and dissolved in dH₂O. DNA concentrations, estimated by UV spectrophotometric determinations, were calculated by the formula: concentration (mg/ml) = A₂₆₀ / 20.

Transformation of Competent *E. coli*

To make cells of *E. coli* strains competent, they were grown in 5 ml cultures of YB media (2% Bacto-typtone, 0.5% Bacto-yeast extract, 0.5% MgSO₄, adjusted to pH 7.6 with KOH) at 37°C under aeration until OD₆₁₀ = 0.3. Cells were diluted 1:20 into fresh prewarmed YB media grown under identical conditions until OD₆₁₀ = 0.46, chilled for 5 min on ice, and centrifuged (5 min, 4°C, at 3,200 x g). The supernatants were decanted, cell pellets were resuspended in ice-cold Tbf1 solution (30 mM potassium acetate, 100 mM KCl, 10 mM CaCl₂, 50 mM MnCl₂, and 15% [vol/vol] glycerol, adjusted to pH 5.8 with acetic acid), and chilled on ice for 5 min. Cells were again pelleted by centrifugation, supernatants decanted, cell pellets resuspended in ice-cold Tbf2 (10 mM MOPS, 75 mM CaCl₂, 10 mM KCl, and 15% [vol/vol] glycerol, adjusted to pH 6.5 with KOH), and chilled on ice for 15 min. Aliquots of frozen competent cells were stored at -70°C following freezing in an isopropanol/dry ice bath. Plasmid transformations with competent cells were performed as described (53, 95, 101) by gently mixing a 50 µl aliquot of thawed competent cells and 1-3 µl of plasmid DNA (1-10 ng) in a 15 ml round-bottom polypropylene tube. Contents were incubated on ice for 5 min, followed by a heat-shock pulse at 42°C for 60 s, and placed on ice for 2 min. LB supplemented with 0.25% glucose was added to transformed cells. These cells were allowed to express their
respective antibiotic resistance(s) during a 1 h incubation before being plated onto
selective media and incubated ON. Chromosomal transformations were performed with
chromosomal DNA (~0.1 µg - 1.0 µg) from *nuo* mutant cells and transformed into
competent cells.

**Generalized Transduction**

Generalized transduction involved the use of P1kc (101). Donor phage lysates
were prepared by inoculating single colonies into 5 ml of LB and grown with aeration at
37°C ON. 50 µl of ON cultures were inoculated into two tubes containing fresh LB
media (5 ml, each supplemented with 0.2% D-glucose and 5 mM CaCl₂); to one tube no
phage was added (control); and to the other 100 µl of P1 phage lysate (grown on the
ATCC strain K-12) was added. Growth with aeration at 37°C continued until lysis was
complete. Lysates were clarified by the addition of 100 µl chloroform followed by
centrifugation at 3,000 x g for 15 min. Recipients cells were prepared by diluting ON
cultures into fresh LB media (supplemented with 0.1% D-glucose and 10 mM CaCl₂) with
an initial OD₆₁₀=0.02-0.04. These cultures were aerated at 37°C until they reached an
OD₆₁₀=0.4-0.6. Cells were then infected with 0, 10, or 100 µl donor lysate and incubated
without aeration for 20 min at 37°C. To prevent phage readsorption, 200 µl of 1 M
sodium citrate was added, the cells centrifuged, and the resultant pellets resuspended in
500 µl LB supplemented with 250 mM sodium citrate. The cells were then incubated at
37°C for 1 h to allow for expression, centrifuged, resuspended in 100 µl of 1M sodium
citrate, and plated onto appropriate selection media. Transductions involving donor
and/or recipient strains that carried the polA(Ts) allele were grown at the permissive temperature of 32°C.

**Cloning and Restriction Endonuclease Analysis**

Restriction endonuclease digestions of chromosomal or plasmid DNA were performed according to the manufacturers' specifications and as described (95). Digested DNA fragments were electrophoretically separated on agarose gels 0.8%-2.0% (wt/vol) in 1X TAE buffer (40 mM Tris-acetate, 1 mM EDTA). Fragments < 500 bp were purified with the Wizard PCR Prep Purification System (Promega); larger fragments were purified by treating agarose pieces with 1.5 vol of 6 M NaCl followed by incubation at 50°C for 15 min, and purification of DNA with the Promega Wizard Miniprep Purification System. Cohesive-end ligation of DNA fragments was performed as described (95). Vector arms were usually dephosphorylated at 5' termini using calf intestinal alkaline phosphatase (Gibco-BRL) according to manufacturer's instructions.

**Polymerase Chain Reaction**

Either plasmid DNA or chromosomal DNA was used as a template for the polymerase chain reaction (PCR). To amplify products 200-bp to 1.2-kb in length, each PCR, performed in a 100 µl volume, contained template (~10 ng plasmid DNA or ~100 ng chromosomal DNA), primers (0.1 m. each), deoxynucleoside triphosphates (0.2 mM each), MgCl₂ (2.0 mM), PCR buffer (20 mM Tris-HCl and 50 mM KCl), and 2.0 U Taq DNA polymerase (Gibco-BRL). The PCR was performed in an Perkin-Elmer 9600
Thermal Cycler (Foster City, CA) for 40 cycles. Each cycle consisted of denaturation at 94°C for 30 sec, annealing for 1 min at 55°C, and extension for 1 min at 72°C. A final 5 min extension step at 72°C was employed.

Long-distance PCR, using the Advantage KlenTaq Polymerase Mix (Clontech Laboratories, Palo Alto, CA), was employed to amplify fragments >1.2-kb. Each PCR, performed in a 50 μl volume, contained ~100 ng chromosomal DNA as template, primers (400 nM each), dNTPs (0.25 mM each), KlenTaq PCR reaction buffer [40 mM Tricine-KOH, pH9.2 @ 25°C; 15 mM KOAc; 3.5 mM Mg(OAc)₂; 75 μg/ml BSA; and 1X KlenTaq Polymerase Mix (KlenTaq-1 DNA polymerase, DeepVent DNA polymerase, and TaqStart antibody (1.1 μg/μl) in storage buffer)]. The PCR was carried out in a Perkin-Elmer 9600 Thermal Cycler using the following profile: 1 cycle, 94°C, 1 min; 30 cycles, 95°C 15 s, 55°C 30 s, 68°C 6 min; 1 cycle, 68°C, 6 min; soak at 4°C.

RNA Extraction

Total cellular RNA was extracted from cells grown in TB at 32°C to mid-exponential phase (OD₆₁₀ = 0.35-0.4) using the RNeasy mini prep kit columns according to the manufacturer’s instructions (QIAGEN). Prior to quantification, RNA samples were subjected to DNaseI (1U/μg; RQ1 RNase-free DNaseI; Promega) and purified using RNeasy columns. The RNA concentration and purity were estimated by measuring the A₂₆₀ and A₂₈₀ of the solution on a DU460 spectrophotometer (Beckman).
Dot Blot and Northern Blot Analyses

For dot blot analysis, 0.5 µg RNA from each strain was directly transferred to multiple GeneScreen Plus nitrocellulose membranes (NEN Life Science Products, Boston, MA), using the Schleicher and Schuell (Keene, NH) dot blot apparatus according to the manufacturers’ instructions. The RNA was both heat- and UV-fixed to the membranes. The resultant RNA dot blots were prehybridized at 42°C in the presence of denatured, salmon or herring sperm DNA for 2-6 h in a buffer comprised of 5X SSC, 50% (w/v) deionized formamide, 5X Denhardt’s solution, 1% SDS, and 10% dextran sulfate (Na salt). Hybridization was performed for 16-28 h in the same buffer, except that the nonhomologous DNA was excluded. DNA probes labeled with [α- 32P]dCTP using the RTS RadPrime DNA labeling system (Gibco BRL) were added to the hybridization solution following denaturing. The membranes were washed as per the manufacturer’s instructions before autoradiography with Kodak XAR film (New Haven, CT) at -70°C for 8 days.

For northern blot analysis, 5 µg of total RNA was denatured at 65°C for 15 min in the presence of 2M formaldehyde, 50% formaldehyde, and 1X MOPS then electrophoresed on a 1% agarose gel containing 2M formaldehyde and 50% formamide in 1X MOPS running buffer. 3 µl of 1 mg/ml EtBr was added directly to each RNA sample prior to electrophoresis. Following electrophoresis, the RNA was transferred by capillary action to a GeneScreen Plus nitrocellulose membrane (NEN Life Science Products) as described by the manufacturer. The RNA was both heat- and UV-fixed to the membrane. Prehybridization, hybridization, washing, probing, and autoradiography were all
performed as described for the RNA dot blots. Stripping of the probe was performed by boiling the membranes in a solution of 2% SDS and 2X SSC for 10 min. Stripping was verified by autoradiography.

**SDS-PAGE and Immunoblot Analysis**

Total whole cell lysate protein (100 µg) and/or purified complex I (a gift from T. Friedrich) were loaded onto 7.5% polyacrylamide gels in the presence of 0.1% SDS (65) and subjected to a voltage of 300 V, using a SE600 protein gel apparatus (Hoefer Scientific Instruments, San Francisco, CA), until the loading dye was about 1 cm from the bottom. The proteins were transferred electrophoretically overnight to 0.45 µm nitrocellulose membranes using a Trans-Blot Cell apparatus (Bio-Rad Laboratories, Richmond, CA). The membranes were blocked with 5% w/v nonfat dried milk in TBST, composed of 20 mM Tris-HCl (pH 7.6), 150 mM NaCl, and 0.1% Tween 20, for 2 h at room temperature. They were washed for 5 min in TBST before being subjected to sequential incubation with rabbit anti-*E. coli* complex I polyclonal antibody #2409 (also a gift from T. Friedrich) for 2 h at room temperature and goat anti-rabbit IgG (heavy and light chain specific) alkaline phosphatase-conjugated antibody at appropriate dilutions for 2 h at room temperature. Finally, they were washed three times for 5 min each in TBST between each incubation step. Color development was achieved with nitroblue tetrazolium and 5-bromo-4-chloro-3-indoylphosphate as described (55).
Fig. 6. Operon fusion vector. pHF9, a multi-copy nuoPA'::lacZYA transcriptional (operon) reporter fusion constructed from pRS415 (102). The 443-bp nuo insert consists of the nuo promoter (114) and the proximal third of nuoA. ApR, ampicillin resistance; Tt4, transcriptional terminator; trpA', trp operon sequence; lacZYA, lac operon including its translational machinery but missing the lac promoter.

Reporter Fusion Construction and β-galactosidase assays

For studies examining the role of nuo mutations on nuo promoter activity, a multi-copy nuoPA'::lacZYA reporter fusion was constructed by subcloning a 443-bp fragment of plasmid pNUO2.3 (114) that encompasses the nuo promoter (nuoP) and the proximal third of the nuoA gene into pRS415, an operon (transcriptional) fusion vector (102). The resultant plasmid, pHF9 (Fig. 6), was transformed into both wild-type and nuo mutant cells and the β-galactosidase activity of the transformants quantified as a measure of nuo promoter activity (17, 102). β-galactosidase activity was determined in an ONPG assay according to the procedure of Miller (78) and the Sigma Chemical Co. (St. Louis, MO), except that the cells were disrupted by sonication, centrifuged at 12,000 rpm for 5 min, and the initial rate of reaction was measured. The protein concentration was determined by the BCA reagent method (103). β-galactosidase activity is expressed in terms of specific activity (sp act; U/mg, where U=µmol ONP formed/min). Determination of both protein concentration and β-galactosidase activity was performed using a DU640
Fig. 7. Protein fusion vectors. pHF13 and pHF14, multi-copy nuo::lacZYA translational (protein) reporter fusions constructed from pRS414 (102). pHF13, the short fusion, is analogous to pHF9 in that it contains a 443-bp nuo insert consisting of the nuo promoter and the proximal third of nuoA. However, pHF14, the long fusion, contains a 992-bp nuo insert consisting of the nuo promoter, the entire nuoA gene, and the proximal third of nuoB. ApR, ampicillin resistance; T1₄, transcriptional terminator; trpA', trp operon sequence; lac'ZYA, lac operon excluding its transcriptional and translational machinery and the first seven codons of lacZ.

spectrophotometer (Beckman). For studies involving nuo::lacZYA protein (translational) fusions, two multi-copy reporter constructs were made. These constructs differed from pHF9, the operon fusion described previously. Both protein fusions lack the lac translational machinery present in the operon fusion: instead they utilize nuo translational machinery. One protein fusion, analogous to the operon fusion, was constructed by subcloning a 443-bp fragment of plasmid pNUO2.3 (114) that encompasses the nuo promoter (nuoP) and the proximal third of the nuoA gene into pRS414, a protein (translational) fusion vector (102). The second fusion was constructed by subcloning a larger 992-bp fragment of pNUO2.3 that encompasses the nuo promoter (nuoP), all of the nuoA gene, and the proximal third of the nuoB gene into pRS414. The resultant multi-
copy fusions were designated pHF13 (the short fusion) and pHF14 (the long fusion), respectively (Fig. 7).

**Expression Constructs**

The genes *nuoH-N* were amplified from the chromosome of CP875 (wild-type) by PCR using the high fidelity polymerase mixture Advantage KlenTaq Polymerase Mix (Clontech Laboratories) and the primer G15 (5'-CACGCGTCTACATTAGTTACG-3'), which lies 157-bp upstream of the *nuoH* start site, and primer N3 (5'-TAACGCTCTAGATCTATCTAC-3'), which lies 86-bp downstream of *nuoN*. The resultant amplification product was 7539-bp and contained unique *XbaI* sites flanking the ends which were introduced by the primers. The *XbaI* sites were used to clone the product into the expression vector pBAD33 (51) (Fig. 8). Since it is a pACYC184-derived vector, pBAD33 is compatible with ColE1-derived vectors (such as pRS415, the operon fusion vector). The cloning was not directional, therefore two constructs were generated: one in which the *nuo* genes were oriented such that they would be transcribed from the P$_{BAD}$ promoter and one in which the *nuo* genes were oriented opposite the P$_{BAD}$ promoter. These constructs were designated pHF78 and pHF79, respectively. Either pBAD33 vector, pHF78, or pHF79 were transformed into wild-type cells (CP875) along with pHF9 (the *nuoPA*'::lacZYA operon fusion). Single transformants and double transformants were selected for by the appropriate antibiotic resistance(s) on LB plates containing the chromogenic indicator X-gal (40 µg/ml) and L(+)-arabinose (0.05%) as an inducer of P$_{BAD}$. Subsequently, the transformants were grown in TB at 32°C in the
Fig. 8. pBAD33:nuo expression constructs. pHF78 and pHF79, multi-copy expression plasmids constructed from the pACYC184-derived, arabinose-inducible vector, pBAD33 (50). araC, co-inducer of the ara operon; P_{BAD}, the arabinose-inducible promoter of the ara operon; 5S and rrnB_{T12}, parts of the 5S rRNA and the strong rrnB terminators, respectively; bla', truncated β-lactamase gene; cat, chloramphenicol acetyltransferase gene encoding Cm resistance. The 7539-bp nuoH-N insert was cloned either in the same orientation as P_{BAD} or in the opposite orientation resulting in pHF78 and pHF79, respectively.

presence of antibiotics and 0.05% arabinose, until they reached mid-exponential phase (OD_{610}=0.35-0.4). The β-galactosidase activity of these transformants was quantified in an ONPG assay (described above) as a measure of nuo promoter activity (17, 102).

**Computer Analysis**

Protein sequence analysis was performed using the BestFit, Gap, Publish, and PeptideSort programs within the Wisconsin Package software (version 8.1) of the Genetic Computer Group (42). Protein sequence motifs were identified by searching the PROSITE Dictionary of Protein Sites and Patterns, also part of the Wisconsin Package. Autoradiographs of the RNA dot blots, northern blots, and the photograph of the RNA agarose gel were scanned using DeskScan II (56) and the images were compiled in PowerPoint (77).
CHAPTER III

EXPERIMENTAL RESULTS

Polarity of nuo Mutants

To determine whether each nuo mutation exerts a polar effect upon transcription of downstream genes, we performed RNA dot blot analysis. Additionally, this technique allowed us to confirm the construction of each mutation beyond the restriction analysis, Southern blotting, and/or PCR that had been used to first identify each mutation. From cells carrying either the wild-type nuo locus (strains CP875 and CP366) or the mutant alleles nuoB::Km (AJW844), nuoB-C::Cm (AJW853), nuoF::miniTnI0Cm (AJW851), Δ(nuoF-L)-1 (CP938), nuoG::TnI0-1 (CP910), ΔnuoG1 (AJW931 and AJW1516), nuoG2 (AJW1470 and AJW1517), nuoH::Km (AJW845), nuoI::Km (AJW846), nuoM::miniTnI0Cm (AJW852), or nuoN::Km (AJW847), we purified total cellular RNA, transferred that RNA directly to multiple nitrocellulose membranes, and then hybridized each membrane with one of ten nuo probes (Fig. 9). Each probe hybridized to RNA extracted from wild-type cells (lanes 1 and 2). Similarly, all probes complementary to sequences located upstream of the reported location of each insertion hybridized to RNA extracted from mutant cells. In contrast, probes complementary to sequences downstream of each insertion hybridized poorly, if at all. For example, the upstream probes nuoA, nuoB, nuo 'CDE', nuoD, and nuoF, but not the downstream probes CTR,
Fig. 9. Autoradiograph of RNA dot blots. Total RNA was purified from wild-type or *nuo* mutant cells, 0.5 µg transferred directly to multiple nitrocellulose membranes, and probed with the *nuo* genes or fragments indicated on the left. Lanes: 1, wild-type (strain CP875); 2, wild-type (CP366); 3, *nuoB::Km* (AJW844); 4, *nuoB-C::Cm* (AJW853); 5, *nuoF::miniTnl0Cm* (AJW851); 6, Δ(*nuoF-L*)-1 (CP938); 7, *nuoG::Tnl0-1* (CP910); 8, Δ*nuoG1* (AJW931); 9, *nuoH::Km* (AJW845); 10, *nuoI::Km* (AJW846); 11, *nuoN::Km* (AJW847). The hybridization pattern for the *nuoM::miniTnl0Cm* strain (AJW852) was identical to that for the *nuoN::Km* strain (AJW847; lane 11); the hybridization pattern for the Δ*nuoG1* strain AJW1516 was identical to that of AJW931 (lane 8). Every probe hybridized to RNA from both *nuoG2* strains, AJW1470 and AJW1517 (data not shown).
nuoH, nuol, and nuo 'MN3', hybridized with RNA from the nuoG::Tnl0-1 strain (lane 7). The faint signal observed with the nuoG probe likely resulted from its hybridization with RNA encoded by the portion of nuoG located upstream of the insertion. We observed similar hybridization patterns with RNA extracted from every nuo mutant constructed by insertion (lanes 4, 5, 9, 10, and 11), with one exception: RNA from the nuoB::Km mutant (lane 3) hybridized with the downstream probe nuo 'CDE'. The location of the nuoB::Km mutation (strain AJW844) relative to that of mutations nuoB-C::Cm and nuoF::miniTnl0Cm was verified genetically (97.4% and 70.8% co-transformation frequency, respectively) and confirmed by Southern blot analysis (data not shown).

Based on these two results, we conclude that the insertion does reside within the nuoB open reading frame. The signal we observed with nuoB::Km RNA when probed with the nuo 'CDE' probe is not likely due to non-specific hybridization with any portion of nuoA or nuoB; if that were the case, we would have expected a signal with every RNA. Instead, it is possible that the signal is a result of contamination of the RNA. In contrast to nuo mutants carrying insertions, those harboring the deletion alleles Δ(nuoF-L)-1 (lane 6) or ΔnuoG1 (AJW931, lane 8) or those carrying the tandem duplication nuoG2 (AJW1470 and AJW1517, data not shown) exhibited different patterns. With these three mutations, probes complementary to sequences both upstream and downstream of the respective mutations hybridized. The CTR, nuoG, nuoH, and nuol probes did not hybridize with RNA isolated from the Δ(nuoF-L)-1 strain (lane 6), presumably because those genes had been deleted from the chromosome. Likewise, the CTR probe did not hybridize with RNA isolated from the ΔnuoG1 strain (AJW931, lane 8).
On the basis of these observations, we conclude that each insertion used to construct a nuo mutant exerted a polar effect upon downstream transcription. In contrast, the two deletions and the tandem duplication each exerted no polar effect. Moreover, the ΔnuoG1 and nuoG2 alleles represent the only non-polar mutations constructed within a single nuo gene.

Northern blot analysis

In an effort to examine individual nuo transcripts, we performed northern blot analysis on total RNA isolated from either wild-type or Δ(nuoF-L)-1 mutant cells. We chose to examine the transcription of two genes, nuoA and nuoH. We anticipated that if there were a single nuo transcript, then both probes would identify that transcript. Likewise, if there were multiple nuo transcripts, then we may be able to identify some or all of them using these two probes. Additionally, by comparing the hybridization patterns obtained with the nuoA probe versus the nuoH probe, we could determine whether the signal we observed was specific or non-specific hybridization. Finally, we used the unrelated acsA probe to serve as a positive hybridization control.

RNA was extracted from wild-type cells (CP875) at OD$_{610}$=0.25, 0.50, and 0.75 and from Δ(nuoF-L)-1 mutants cells (CP938) at OD$_{610}$=0.50. A photograph of the RNA agarose gel following electrophoresis is shown in Fig. 10. Both the 16S and 23S rRNA bands are clearly visible for each sample. The RNA was transferred to a nitrocellulose membrane overnight by capillary action then heat- and UV-fixed to the membrane. The
Fig. 10. RNA agarose gel. Cells were grown in TB at 32°C to the indicated optical density at which point total RNA was extracted as described in Materials and Experimental Methods. 5 μg of total RNA from wild-type cells (CP875) and Δ(nuoF-L)-1 mutant cells (CP938) was denatured at 65°C for 15 min in the presence of 2M formaldehyde, 50% formaldehyde, and 1X MOPS then electrophoresed on a 1% agarose gel containing 2M formaldehyde and 50% formamide in 1X MOPS running buffer. 3 μl of 1 mg/ml EtBr was added directly to each RNA sample prior to electrophoresis. The RNA marker sizes are indicated on the left (Gibco-BRL). The 23S (3.7-kb) and 16S (1.7-kb) rRNA bands are indicated on the right. Not shown: A nuoA DNA fragment and a nuoH DNA fragment were run on the gel to serve as positive hybridization controls during northern blot analysis.
membrane was probed with a \textit{nuoA} probe, stripped, probed with a \textit{nuoH} probe, stripped, and probed with an \textit{acsA} probe.

The \textit{nuoA} probe hybridized in a diffuse pattern with RNA from both wild-type and \(\Delta(\text{nuoF-L})\)-1 mutant cells (Fig. 11A). The signal appeared to center on the rRNA bands and no signal was observed that exhibited slower mobility than the 23S rRNA band. The \textit{nuoH} probe also hybridized in a diffuse pattern centered around the rRNA bands (Fig. 11B), but it did not hybridize to RNA extracted from the \(\Delta(\text{nuoF-L})\)-1 mutant. This is expected since the \textit{nuoH} gene is deleted from the chromosome in this mutant. The lack of a \textit{nuoH} hybridization signal in the \(\Delta(\text{nuoF-L})\)-1 mutant indicates a lack of non-specific hybridization with wild-type RNA. We observed that the hybridization signal from RNA extracted from wild-type cells at OD\(_{610}\) =0.75 was less intense than the signals from RNA extracted at OD\(_{610}\) =0.25 or 0.5. Without an additional loading control, we can not draw any conclusions about whether the difference in signal intensities is significant.

To verify the integrity of total RNA, we probed the blot with an internal fragment of the \textit{acsA} gene that corresponded approximately in size to the \textit{nuoA} probe (Fig. 11C). A single transcript of about 2.1-kb was observed. This result had been obtained previously (Kumari and Wolfe, manuscript in preparation). Furthermore, when we used \textit{nuoA} to probe a different blot (one that previously had displayed the \textit{acsA} transcript; a gift from S. Kumari), we again observed the diffuse hybridization pattern observed with the first blot (Fig. 11D).
Fig. 11. Autoradiographs of northern blots. RNA was transferred electrophoretically from the agarose gel (Fig. 10) to a nitrocellulose membrane. The membranes were stripped between each probing. Probes are indicated in the gray boxes. (A) \( \text{nuoA} \) probed membrane, (B) \( \text{nuoH} \) probed membrane, (C) \( \text{acsA} \) probed membrane. The blots in A-C were exposed to film for 8 days. (D) \( \text{nuoA} \) probed membrane; a different membrane was used, one that had been probed previously with \( \text{acsA} \) and stripped. The blot was exposed for 5 days. Wild-type, strain CP875; \( \Delta(\text{nuoF-L}) \)-1, CP938. \( \Delta\text{acsA}::\text{Km} \) mutant, AJW803. \( \text{OD}_{610} \), the point at which the RNA was extracted from the respective cells. The 23S and 16S rRNA bands are indicated to the right of each blot.
These results suggest that i) the hybridization signals we observed are specific for
*nuo*, ii) the hybridization pattern we observe with the *nuoA* and *nuoH* probes is a
phenomenon associated with *nuo* and is not due to non-specific hybridization, but iii) the
*nuo* transcript(s) does not appear to be stable under the conditions tested.

**Phenotypes of *nuo* Mutants**

To test whether i) the newly constructed *ΔnuoG1* and *nuoG2* mutants exhibited
the previously described Nuo- phenotypes and ii) all of the isogenic *nuo* mutants
exhibited the same phenotypes, we characterized the mutants by subjecting them to a
series of tests, each shown previously to distinguish wild-type cells from those of *nuo*
mutants (4, 8, 23, 24, 67, 68, 91). On the basis of the five phenotypic analyses, we
conclude that all of the *nuo* mutants tested exhibit a pleiotropic Nuo- phenotype.
Additionally, the data suggests that the *nuoN* mutant possesses a subassembled,
cytoplasmic peripheral fragment (NDF).

First, we inoculated cells carrying either the wild-type *nuo* locus (strain CP366) or
the mutant alleles *nuoG::Tn10-1* (CP932) or *ΔnuoG1* (AJW931), at the center of TB
swarm plates, incubated those plates at 32°C until the outer, serine ring had reached the
edge, and examined them for the presence or absence of the inner, aspartate ring. Wild-
type cells formed both rings (Fig. 12A). In contrast, the *nuoG::Tn10-1* mutant
Fig. 12. Photographs of the swarms produced by the (A) wild-type strain (CP366), (B) nuoG::Tnl0-1 mutant (CP932), (C) ΔnuoG1 mutant (AJW931), and (D) the ΔnuoG1 integrant (AJW932). Cells were inoculated at the center of a TB swarm plate containing 0.25% agar, and the plates incubated at 32°C until the outer, serine ring reached the edge of the swarm plate.

(Fig. 12B), the ΔnuoG1 mutant (Fig. 12C), and all other nuo mutants failed to form the inner ring (Table 4).

Second, we inoculated TB with wild-type cells (strain CP875) or those that carry the mutant alleles Δ(nuoF-L)-1 (CP938), nuoG::Tnl0-1 (CP910), ΔnuoG1 (AJW1516), or nuoG2 (AJW1517), aerated the cultures at 32°C, and monitored their growth as a function of optical density (Fig. 13). Prior to mid-exponential phase, both wild-type and mutant cells grew at similar rates. Whereas wild-type cells continued relatively rapid
**Fig. 13. Tryptone broth growth curve.** Cells were grown in TB at 32°C. Growth was monitored as a function of optical density versus time. The OD$_{610}$ of the wild-type strain CP875 (■), the Δ(nuoF-L)-1 strain CP938 (Δ), the nuoG::Tn10-1 strain CP910 (○), the ΔnuoG1 strain AJW1516 (▽), and the nuoG2 strain AJW1517 (◊). The mean of six independent experiments is plotted for each strain. The error bars represent ±SEM.

growth beyond OD$_{610}$=0.3, mutant cells abruptly reduced their growth rate. Regardless of genetic background, all other *nuo* mutants exhibited similar behavior (Table 4).

Third, to examine the cells growth on acetate as the sole carbon source, we inoculated M63 minimal plates supplemented with 25 mM acetate or 0.25% glucose with wild-type cells (strain CP875) and those carrying the mutant alleles *nuoG::Tn10-1* (CP910), Δ*nuoG1* (AJW1516), or *nuoG2* (AJW1517), incubated those plates at 32°C for 55.5 h before photographing them. Relative to wild-type cells, mutant cells formed very
small colonies on M63 acetate plates (≤0.5 mm compared to ≥1.0 mm for wild-type cells) (Fig. 14). In contrast, all cells formed equally-sized colonies (~1.0 mm) on M63 glucose plates. All other nuo mutants tested formed equivalent colony sizes (Table 4).

Fourth, complex I activity was examined in selected mutants by measuring the NADH ferricyanide (FeCN) and d-NADH FeCN activities in their membrane fractions. These experiments were performed by Thorsten Friedrich and colleagues at the Heinrich-Heine-Universität Düsseldorf. Whereas membrane fractions from wild-type cells

![Fig. 14. Growth on acetate. Photographs of colonies produced by the wild-type strain CP875, the nuoG::Tn10-1 strain CP910, the ΔnuoG1 strain AJW1516, and the nuoG2 strain AJW1517. Cells were inoculated on minimal media (M63) plates supplemented with 25 mM sodium acetate (pH 7.0) and the plates were incubated at 32°C for 55.5 h.](image-url)
exhibited both these complex I activities, membrane fractions of the mutant cells
exhibited much lower levels of these activities (10) (Table 4). The activities were not
completely abolished in the *nuo* mutants presumably because the second NADH
dehydrogenase, NDH-II, also reacts with both substrates (37).

Finally, using electron-paramagnetic resonance spectroscopy (EPR) analysis that
detects Fe-S centers of complex I in the membrane fraction of wild-type cells (67, 68,
114), Friedrich *et al.* tested for the presence of subcomplexes of complex I in the mutants.
The analysis revealed no detectable amounts of complex I or subcomplexes of complex I
in the membrane fractions or cytoplasm of any of the mutants tested with one notable
exception: EPR analysis detected Fe-S centers in the cytoplasm of the *nuoN* mutant, but
not in its membrane fraction (10) (Table 4). The presence of Fe-S centers in the
cytoplasm of *nuoN* mutants was confirmed by the purification of the peripheral fragment
(NDF) from those cells which was subsequently shown to resemble the NDF of purified
complex I in both enzyme activity and EPR spectroscopy (10).

**Complementation of the Δ*nuoG1* and *nuoG2* Alleles**

We tested for complementation *in cis* (32) with multiple, independent isolates of
strains that carry both the wild-type *nuoG* and the mutant Δ*nuoG1* alleles (strains
AJW932 and AJW1459) or that carry both the wild-type *nuoG* and the mutant *nuoG2*
alleles (AJW1472) on their chromosomes by scoring each strain for their ability to
produce the inner ring in swarm assays (Fig. 12D; Table 4). Multiple, independent
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<th>swarm plate</th>
<th>TB growth curve</th>
<th>Growth on M63 + 25 mM acetate</th>
<th>NADH/dNADH FeCN curve analysis</th>
<th>EPR spectroscopy analysis</th>
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Strain | nuo mutation | swarm plate | TB growth curve | Growth on M63 + 25 mM acetate | NADH/dNADH FeCN activity | EPR spectroscopy analysis
--- | --- | --- | --- | --- | --- | ---
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AJW1580 | nuoB::Km nuoF::miniTn10Cm | – | – | – | ND | ND
AJW1581 | nuoB::Km nuoB-C::Cm | – | – | – | ND | ND
AJW1582 | ΔnuoG1 nuoH::Km | – | – | – | ND | ND
AJW1583 | ΔnuoG1 nuoI::Km | – | – | – | ND | ND
AJW1584 | nuoG2 nuoH::Km | – | – | – | ND | ND

*a, the presence of an inner, aspartate ring; –, the absence of the inner ring.
*b, no growth defect; -, growth defect.
*c, good growth (>1.0 mm colonies); -, poor growth (<0.5 mm colonies).
*d, presence of the activities in membrane fractions; -, decrease in these activities.
*e, the presence of Fe-S centers characteristic of complex I; -, lack of detectable Fe-S centers.
*f, ND, not done for this study.
*g, Strain background was Ace- prior to introduction of nuo mutation(s).
*h, Mutation analyzed in a different, but related, background.

isolates of each strain formed an inner, aspartate ring despite the fact that vector sequence interrupts the partially duplicated nuo locus in these strains (see Fig. 5). Additionally, strains AJW932, AJW1459, and AJW1472 did not exhibit the TB growth defect (Table 4). Since these strains exhibited these two Nuo+ phenotypes, we conclude that the ΔnuoG1 and nuoG2 alleles can be complemented and that the mutant alleles are recessive.
Translational Analysis

We examined the ability of wild-type and *nuo* mutant cells to synthesize the NuoCD and NuoG subunits (67), using a polyclonal antibody directed against purified complex I (#2409). The NuoCD subunit has been identified as a single polypeptide (a fusion protein) in *E. coli* (41). This finding is consistent with a study of the bacterium *Buchnera aphidicola* which described a protein homologous to NuoC at its N-terminus yet homologous to NuoD at its C-terminus (20). However, due to a sequence discrepancy, it is not yet clear the mechanism for such a fusion protein. There is possibility that the proposed *nuoC* stop TGA codon may code for the amino acid selenocysteine or that the stop codon is simply out of frame and therefore only a single *nuoCD* open reading frame exists.

We identified the bands that correspond to NuoCD and NuoG by comparing the pattern of bands from purified complex I (67) (Fig. 15A and B, lane 1) to those of a mixture of purified complex I and whole cell lysate from wild-type cells (strain CP366) (Fig. 15A and B, lane 2) to whole cell lysates from wild-type cells alone, strains CP366 (Fig. 15A and B, lane 3) and CP875 (Fig. 15A only, lane 4). We examined both wild-type strains because the mutants were constructed in the two backgrounds. We failed to detect NuoCD only in strains that carry an insertion mutation upstream of *nuoC* (Fig. 15A only, lanes 5 and 6), but detected NuoCD in all other strains (Fig. 15A, lanes 3, 4, and 7-14; Fig. 15B, lanes 3-9). Similarly, we failed to detect NuoG in strains that either lack *nuoG* or harbor an insertion mutation upstream of or within *nuoG* (Fig. 15A, lanes 5-9; Fig. 15B, lanes 4 and 5), but detected NuoG in all other strains (Fig. 15A, lanes 3, 4,
Fig. 15. Immunoblot analysis using *E. coli* anti-complex I polyclonal antibody following SDS-PAGE (7.5% polyacrylamide) and transfer to nitrocellulose. Molecular weight markers are indicated on the right; the NuoG and NuoCD subunits are identified on the left. (A) Analysis of whole-cell lysates prepared from wild-type or *nuo* polar mutant cells. Lanes: 1, purified complex I; 2, purified complex I mixed with wild-type lysate (strain CP366); 3, wild-type (CP875); 4, wild-type (CP366); 5, *nuoB::Km* (AJW844); 6, *nuoB-C::Cm* (AJW853); 7, *nuoF::miniTn10Cm* (AJW851); 8, Δ(*nuoF-L-1*) (CP938); 9, *nuoG::Tn10-1* (CP910); 10, *nuoH::Km* (AJW845); 11, *nuoI::Km* (AJW846); 12, *nuoM::miniTn10Cm* (AJW852); 13, *nuoN::Km* (AJW847). Cells were grown with aeration in TB at 32°C until cultures reached mid-exponential phase (OD$_{610}$=0.35-0.4). 100 µg whole-cell lysate loaded in each lane. (B) Analysis of whole-cell lysates prepared from wild-type or *nuoG* mutant cells. Lanes: 1, purified complex I; 2, purified complex I mixed with wild-type whole-cell lysate (CP366); 3, wild-type (CP366); 4, Δ(*nuoF-L-1*) (CP938); 5, *nuoG::Tn10-1* (CP910); 6, Δ*nuoG1* (AJW931); 7, *nuoG2* (AJW1470). Cells were grown as in Panel A. 100 µg whole cell lysate loaded in each lane.
Fig. 1A. Precursor NuoG and NuoCD of the wild-type strain. The mass spectrometry analysis of the translation products from the wild-type strain revealed two bands at 97kDa and 68kDa.

Fig. 1B. Precursor NuoG and NuoCD of the ΔnuoG strain. The translation products from the ΔnuoG strain showed a single band at 68kDa.
11-14; Fig. 15B, lane 3). Cells that carry the alleles ΔnuoG1 (AJW931 (Fig. 15B, lane 6) and AJW1516 (data not shown)) or nuoG2 (AJW1470 (Fig. 15B, lane 7) and AJW1517 (data not shown)) synthesized proteins that exhibited faster and slower mobility,

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<th>NuoG subunit (N → C-terminus)</th>
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</tbody>
</table>

Fig. 16. Putative NuoG peptides. Length and molecular mass (Mₚ) of translation products from the wild-type and mutant nuoG alleles as predicted by PeptideSort (41). The C-terminal region (CTR) of NuoG (gray-hatched). The deletion in ΔnuoG1 shifts the remainder of the altered NuoG C-terminus out-of-frame (dark gray). The duplication in nuoG2 shifts the duplicated CTR (black-hatched) and the remainder of the altered NuoG C-terminus (dark gray) out-of-frame.

respectively, compared to the wild-type NuoG. The apparent molecular mass of each variant roughly corresponded to the predicted product of their respective alleles (42) (Fig. 16). In addition, the steady state level of the smaller variant produced by ΔnuoG1 cells (Fig. 15B, lane 6) seemed to be significantly less than that of the full-length NuoG protein produced by the wild-type strain CP366 (Fig. 15B, lane 3). In contrast, the amount of NuoCD protein produced by both strains seemed to be roughly equivalent. On
the basis of these observations, we conclude that the $\Delta nuoG1$ and $nuoG2$ alleles result in the synthesis of altered forms of the NuoG subunit. The NuoG subunit is subject to proteolytic digestion in disrupted cells (33). Perhaps the truncated NuoG subunit resulting from the $\Delta nuoG1$ allele is less abundant as a result of even more rapid proteolytic digestion.

**Effect of $nuo$ Mutations on $nuo$ Promoter Activity**

To examine the effect that $nuo$ mutations exert upon $nuo$ promoter activity, we monitored $\beta$-galactosidase activity from a $nuoPA':::lacZYA$ operon fusion. We transformed cells carrying the wild-type $nuo$ locus (strain CP875) or the mutant alleles $nuoB::Km$ (AJW844), $\Delta(nuoF-L)-1$ (CP938), $nuoG::Tn10-1$ (CP910), $\Delta nuoG1$ (AJW1516), $nuoG2$ (AJW1517), $nuoH::Km$ (AJW845), $nuoI::Km$ (AJW846), $nuoM::Km$ (AJW852), $nuoN::Km$ (AJW847), $\Delta nuoG1 nuoH::Km$ (AJW1582), $\Delta nuoG1 nuoI::Km$ (AJW1583), $nuoG2 nuoH::Km$ (AJW1584), $\Delta nuoG1 nuoM::Cm$ (AJW1567), $\Delta nuoG1 nuoN::Km$ (AJW1568), or $nuoG2 nuoM::Cm$ (AJW1569) with pHF9, a multi-copy plasmid that carries the $nuoPA':::lacZYA$ operon fusion (Fig. 17A) or with its parental vector, pRS415. We grew the resultant transformants in TB at 32°C, harvested and lysed them as the cultures reached mid-exponential phase ($\text{OD}_{610}=0.35-0.4$), and measured their $\beta$-galactosidase activity. The mean sp act±SEM of at least six independent experiments was plotted (Fig. 17B). Wild-type cells transformed with the vector control pRS415 displayed little or no $\beta$-galactosidase activity (sp act of $0.02±0.01$). Relative to pHF9 transformants of wild-type cells, those of the nonpolar mutants $\Delta nuoG1$ and $nuoG2$
exhibited reduced β-galactosidase activity (sp act of 10.3±0.76 vs. 4.83±1.06 and 1.04±0.06, respectively). In contrast, pHF9 transformants of the ΔnuoG1 nuoH::Km, ΔnuoG1 nuoI::Km, and nuoG2 nuoH::Km double mutant strains exhibited significantly higher activities (sp act of 27.50±5.60, 27.11±2.55, and 30.00±1.49, respectively) than did the respective ΔnuoG1, nuoG2, or wild-type transformants. This increase in activity cannot be due merely to the presence of the nuoH::Km or nuoI::Km mutations only since transformants of those single mutants exhibited activities similar to that of the wild-type transformants (10.20±0.68 and 15.74±1.47, respectively). In fact, transformants of all the single mutants tested (except for the ΔnuoG1 and nuoG2 mutants) displayed activities similar to that of the wild-type transformants. The double mutants ΔnuoG1 nuoM::Cm, ΔnuoG1 nuoN::Km, and nuoG2 nuoM::Cm transformed with pHF9 displayed activities only slightly higher than wild-type transformants (16.40±1.56, 18.96±2.04, and 13.52±0.582, respectively). We do not know why there was a difference in the level of activity between the ΔnuoG1 and nuoG2 transformants, importantly their activity is lower than that for wild-type transformants. Moreover, an additional mutation downstream of either the ΔnuoG1 or nuoG2 mutation eliminated any significance difference in those transformants' activities. On the basis of these data, we propose that NuoG participates in the regulation of nuo transcription possibly in conjunction with some other Nuo subunit(s).

Interestingly, nuoG::Tn10 transformant exhibited levels of β-galactosidase activity similar to the wild-type transformant whereas the ΔnuoG1 and nuoG2 transformants displayed lower levels. This difference between the nuoG mutants may be
Fig. 17. Effect of *nuo* mutations on *nuo* promoter activity. (A) pHF9, a multi-copy *nuoPA*::*lacZYA* transcriptional (operon) reporter fusion. Designations as in Fig. 6 legend. (B) β-galactosidase activity assay. Cells were grown in TB at 32°C to mid-exponential phase (OD$_{610}$=0.35-0.4), harvested, lysed by sonication, and centrifuged to separate the cytoplasmic from the membrane fraction. The β-galactosidase activity of the cytoplasmic fractions was quantified as U/mg protein where U= µmol ONP formed/min. The figure is a representation of at least six independent experiments. wt (CP875); *nuoB::Km* (AJW844); Δ(*nuoF-L*)-1 (CP938); *nuoG::Tn10*-1 (CP910); *nuoH::Km* (AJW845); *nuol::Km* (AJW846); *nuoM::miniTn10Cm* (AJW852); *nuoN::Km* (AJW847); Δ*nuoG1* (AJW1516); *nuoG2* (AJW1517); Δ*nuoG1 nuoH::Km* (AJW1582); Δ*nuoG1 nuoI::Km* (AJW1583); *nuoG2 nuoH::Km* (AJW1583); Δ*nuoG1 nuoM::Cm* (AJW1567); Δ*nuoG1 nuoN::Km* (AJW1568); *nuoG2 nuoM::Cm* (AJW1569).
attributed to the polarity of the mutations. In the *nuoG::Tn10* mutant, we saw that all transcription downstream of the mutation is eliminated therefore, none of the genes *nuoG-N* are expressed. However, transcription of downstream genes does not seem to be affected in either the Δ*nuoG1* or *nuoG2* mutant. It is likely that the decrease in β-galactosidase activity in the Δ*nuoG1* and *nuoG2* transformants is due not to the presence of the altered NuoG subunits, rather it is due to the presence of the downstream Nuo subunits unable to properly assemble.

**Effect of Overexpression of *nuo* Genes on *nuo* Promoter Activity**

We observed a significant decrease in expression from the *nuo* promoter only in the Δ*nuoG1* and *nuoG2* mutants transformed with pHF9 and observed a decrease in expression when transcription of *nuoH-N* was abolished by an additional mutation in either *nuoH* or *nuoI*. From our dot blot analysis, we know that the Δ*nuoG1* and *nuoG2* mutants transcribe *nuoH-N*. We assume that the NuoH-N subunits are synthesized in these strains. On the basis of these results, we propose that NuoH-N may affect *nuo* expression by repressing promoter activity. We tested whether the overexpression of *nuoH-N* could decrease expression from the *nuo* promoter. We transformed wild-type (CP875) cells with pHF9 (Fig. 6) alone or in conjunction with the compatible vector pBAD33 or with constructs derived from pBAD33 that contain the *nuoH-N* genes either in the same orientation as the P<sub>BAD</sub> promoter, pHF78, or in the opposite orientation from the P<sub>BAD</sub> promoter, pHF79 (Fig. 8). We plated these transformants on LB X-gal indicator plates containing the appropriate antibiotic(s) and 0.05% arabinose (for P<sub>BAD</sub> induction).
These plates were incubated at 32°C ON at which time the colonies were examined for their color. Blue color signified β-galactosidase activity expressed from pHF9, whereas white color indicated a little or no β-galactosidase activity (78). We quantified the β-galactosidase activity by performing an ONPG assay (described in Materials and Experimental Methods) with transformants grown in TB at 32°C to mid-exponential phase (OD$_{610}$=0.35-0.4). The results of these experiments are presented in a table below.

<table>
<thead>
<tr>
<th>β-galactosidase activity</th>
<th>Wild-type (CP875) cells transformed with:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>nuoPA':::lacZYA</td>
</tr>
<tr>
<td>Color on X-gal:</td>
<td>blue</td>
</tr>
<tr>
<td>sp act (U/mg):</td>
<td>~10.0</td>
</tr>
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</table>

Based on the results we observed on the indicator plates, we conclude that the β-galactosidase activity is decreased in cells transformed with constructs carrying the nuoH-N genes in either orientation (pHF78 or pHF79). This decrease is not merely due to the presence of the pBAD33 vector-backbone, since no decrease was observed in cells transformed with the vector pBAD33 without any nuo insert. The results of the ONPG assay demonstrate that the decrease in β-galactosidase activity is significant. The nuoH-N genes are oriented opposite that of the P$_{BAD}$ promoter in pHF79; their expression may be driven by the araC gene, the cat gene, or perhaps by some internal nuo promoter.
Evidence for an internal promoter between $\textit{nuoG}$ and $\textit{nuoN}$ in \textit{S. typhimurium} has been presented by Archer and Elliott (3).

These results, while inconclusive, are intriguing. We would need to determine whether $\textit{nuoH-N}$ are in fact being expressed in each construct before concluding that it is the overexpression of $\textit{nuoH-N}$ leads to the decrease in $\textit{nuo}$ promoter activity we observed.

**Studies with $\textit{nuo::lacZ}$ Protein Reporter Fusions**

To examine whether NuoA affects the expression from the $\textit{nuo}$ promoter, we constructed two $\textit{nuo::lacZYA}$ protein fusions, one that contained all of $\textit{nuoA}$ and one that did not. These fusions, pHF13 (the short fusion) and pHF14 (the long fusion), are depicted in Fig. 18A. pHF13 is analogous to the operon fusion, pHF9: it contains $\textit{nuoP}$ and the proximal third of $\textit{nuoA}$ cloned into the protein fusion vector pRS414. pHF14 contains a slightly larger insert: it includes $\textit{nuoP}$, all of $\textit{nuoA}$, and the proximal third of $\textit{nuoB}$ cloned into pRS414. The main difference between the two protein fusions is the presence of only a partial $\textit{nuoA}$ gene in pHF13 or the entire $\textit{nuoA}$ gene in pHF14. We assume that NuoA is synthesized from pHF14, but not from pHF13, and therefore the levels of NuoA would be higher in cells transformed with pHF14 than in those transformed with pHF13. We transformed wild-type cells (CP875) with each of the protein fusions or their parental vector, pRS414. We grew the cells in TB at 37°C and measured their $\beta$-galactosidase activity over a growth curve (Fig. 18B). Cells transformed with pHF13 display a higher level of $\beta$-galactosidase activity throughout the
Fig. 18. Expression studies with protein fusions. (A) pHF13 and pHF14, multi-copy nuo::lacZYA translational (protein) reporter fusions. pHF13 contains a 443-bp nuo insert whereas pHF14 contains a 992-bp nuo insert. Designations as in Fig. 7. (B) β-galactosidase activity assay. Wild-type (CP875) cells transformed with vector pRS414 (■), pHF13 (●), or pHF14 (▲). Transformants were grown in TB at 32°C and samples removed at the indicated time points. The cells were lysed by sonication and centrifuged to separate the cytoplasmic from the membrane fraction. The β-galactosidase activity of the cytoplasmic fractions was quantified as U/mg protein where U= µmol ONP formed/min. The mean±SEM of six independent experiments is plotted.
**A**

- **pHF13**
  - EcoRI
  - BamHI
  - **Ap**<sup>R</sup> **T<sub>1</sub>**<sup>nuoPA</sup> 'lacZ lacY lacA

- **pHF14**
  - EcoRI
  - BamHI
  - **Ap**<sup>R</sup> **T<sub>1</sub>**<sup>nuoPAB</sup> 'lacZ lacY lacA

**B**

- Graph showing 
  - **β-galactosidase Activity (U/mg)**
  - **Time (hours)**
  - Data points with error bars
growth curve than do cells transformed with pHF14. These results suggest that the overexpression of nuoA reduces expression from the nuo promoter. This data also substantiates a role for Nuo subunits, in this case NuoA, in the regulation of nuo expression.

The Impact of Sigma Factors on nuo Promoter Activity

Three reports by different research groups describe or make reference to the presence of $\sigma^{70}$-dependent promoter elements, specifically the -10 and/or -35 sequences, within the nuo promoter in both E. coli and S. typhimurium (3, 12, 114). We tested the hypothesis that the nuo promoter is, in fact, $\sigma^{70}$-dependent. We used the nuoPA'::lacZYA operon fusion, pHF9 (Fig. 6 and 17A), to study the effect of the sigma factors $\sigma^{70}$ (the "housekeeping" sigma factor), $\sigma^{5}$ ($\sigma^{38}$; the stationary phase/oxidative stress sigma factor), and $\sigma^{54}$ ($\sigma^{0}$; nitrogen/formate metabolism sigma factor) on nuo promoter activity (Fig. 19).

We transformed the operon fusion, pHF9, into wild-type cells (Strain CP875) or sigma factor mutants (rpoDTs, strain UQ285; rpoS::Km, AJW1015; or rpoN::Tn10-1, AJW1409) and grew the transformants in TB supplemented with the requisite antibiotics at the temperatures indicated. We harvested the cells at the indicated times and measured their $\beta$-galactosidase activity as described previously.

To determine whether the nuo promoter is $\sigma^{70}$-dependent as had been reported previously, we examined nuo expression in an RpoDTs strain. We measured the $\beta$-
galactosidase activity of \textit{rpoD}(Ts) cells (strain AJW1337) transformed with pHF9.

Surprisingly, we observed only a slight decrease in the $\beta$-galactosidase activity exhibited by transformants at the permissive temperature (33°C; $\text{RpoD}^+$) versus those at the restrictive temperature (44°C; $\text{RpoD}^-$) (Fig. 19A).

To determine whether the \textit{nuo} promoter is $\sigma^5$-dependent, we measured the $\beta$-galactosidase activity of wild-type cells (CP875) and \textit{rpoS::Km} cells (AJW1015) transformed with pHF9. We observed only a slight decrease in $\beta$-galactosidase activity in RpoS$^-$ transformants (AJW1015 pHF9) compared to RpoS$^+$ transformants (CP875 pHF9; Fig. 19B). A similar result was achieved in \textit{S. typhimurium}, where a \textit{katF} (\textit{rpoS}) mutation introduced into a strain harboring a single-copy \textit{nuo} promoter fusion resulted in a slight decrease in $\beta$-galactosidase activity (3).

To determine whether the \textit{nuo} promoter is $\sigma^{54}$-dependent, we measured the $\beta$-galactosidase activity of wild-type cells (CP875) and \textit{rpoN::Tn10-1} cells (AJW1409) transformed with pHF9. RpoN$^-$ transformants displayed a significantly reduced level of $\beta$-galactosidase activity relative to the RpoN$^+$ transformants (Fig. 19C).

While these studies are not exhaustive, we conclude that i) $\sigma^{70}$ and $\sigma^5$ play a minor role, if any, in regulating the \textit{nuo} promoter and ii) $\sigma^{54}$ contributes greatly, either directly or indirectly, to expression from the \textit{nuo} promoter.
**Fig. 19. nuo promoter activity in various sigma factor mutants.** β-galactosidase activity assays performed on cells transformed with the multi-copy *nuoPA*::*lacZYA* operon fusion, pHF9 (refer to Fig. 6 and 17A). Transformants were grown in TB at 37°C, unless otherwise stated, and samples removed at the indicated time points. The cells were lysed by sonication and centrifuged to separate the cytoplasmic from the membrane fraction. The β-galactosidase activity of the cytoplasmic fractions was quantified as U/mg protein, where U = µmol ONP formed/min. (A) *rpoD(Ts)* cells (strain AJW1337) transformed with pHF9. Cells were initially grown at 33°C (permissive temperature), then the culture was split and one half was grown at 33°C, the other at 44°C (restrictive temperature). The arrow indicates the time point at which the cultures were split. RpoD+ (33°C; ■), RpoD- (44°C; ○). (B) RpoS+ (wild-type, CP875; ■) or RpoS- (rpoS::Km mutant, AJW1015; ○) cells transformed with pHF9. (C) RpoN+ (wild-type, CP875; ■) or RpoN- (rpoN::TnlO mutant, AJW1409; ○) cells transformed with pHF9.
CHAPTER IV

DISCUSSION

Nuo Phenotypes

We performed a genetic analysis of *nuo*, the *E. coli* locus that encodes the proton translocating NADH dehydrogenase, complex I. Examining physiological, biochemical, and molecular properties of mutants defective in nine of the fourteen *nuo* genes, we demonstrated that a mutation in any one of the *nuo* genes tested causes a complex I deficiency as measured by the inability of the mutant cells to i) form a inner, aspartate ring on chemotaxis swarm plates, ii) grow rapidly in TB beyond mid-exponential phase, iii) use acetate effectively as a sole carbon source, iv) exhibit membrane-associated (d)NADH-FeCN activity, and v) detect membrane-associated Fe-S centers. Since each *nuo* mutant exhibited all five Nuo- phenotypes, we used the swarm assay as a simple and reliable Nuo- screen. The mechanism for this phenotypic defect remains unclear. However, the absence of the inner, aspartate ring on swarm plates may result from the inability to respire aerobically: wild-type cells grown anaerobically in the presence or absence of an electron acceptor also do not form the inner ring (McNamara and Wolfe, unpublished observation).
Transcriptional Analysis

All of the \textit{nuo} insertion mutations tested (\textit{nuoB-C}:\text{Cm}, \textit{nuoF}::\text{miniTn10Cm}, \textit{nuoG}::\text{Tnl0-1}, \textit{nuoH}::\text{Km}, \textit{nuoI}::\text{Km}, \textit{nuoM}::\text{miniTn10Cm}, and \textit{nuoN}::\text{Km}) prevented transcription of downstream genes, as judged by RNA dot blot analyses, with the exception of the \textit{nuoB}::\text{Km} mutation. However, since we verified the location of the mutation genetically and the \textit{nuoB}::\text{Km} mutant cells did not seem to synthesize the NuoCD or NuoG subunit, as demonstrated by immunoblot analysis, the apparent incomplete polarity exhibited by this mutation may be artifactual.

\textit{\Delta{}nuoG1} and \textit{nuoG2} represent the first recessive, nonpolar mutations located within a single \textit{nuo} gene. The RNA dot blot analyses revealed that both deletion mutations (\textit{\Delta{}(nuoF-L)-1} and \textit{\Delta{}nuoG1}) and the duplication mutation (\textit{nuoG2}) do not exert polar effects upon the transcription of downstream genes and complementation analyses demonstrated that both the \textit{\Delta{}nuoG1} and \textit{nuoG2} mutant alleles are recessive: strains that carry both the wild-type \textit{nuoG} allele and either of these mutant alleles on their chromosomes formed inner rings on swarm plates and did not exhibit the TB growth defect. Since these integrant strains exhibited wild-type behaviors, we conclude that the two halves of the \textit{nuo} locus can be expressed independently. Expression of the downstream half of the locus in these strains may result either from a promoter located within \textit{nuoG} or the 3' end of \textit{nuoF} or from a promoter located within the vector.

Evidence for an internal promoter between \textit{nuoG} and \textit{nuoN} in \textit{S. typhimurium} has been presented by Archer and Elliott (3).
We could not identify individual, discrete \textit{nuo} transcripts using northern blot analysis. We believe that this lack of discrete transcript(s) did not occur because the total RNA was degraded during preparation or during transfer: both rRNA bands were intact and a discrete \textit{acsA} transcript was identified. The signal we observed using the both \textit{nuo} probes, however, not a result of non-specific hybridization: we observed a signal when RNA from \(\Delta(\textit{nuoF-L})\)-1 cells was probed with \textit{nuoA} DNA but not when probed with \textit{nuoH} DNA. Unfortunately, the \textit{nuo} transcript(s) does not appear to be stable under the conditions tested. Further analysis of \textit{nuo} transcripts will require the use of alternative techniques such as primer extension, RNase protection, or \textit{in vitro} transcription.

\textbf{Protein Analysis}

This is the first report of immunoblot analysis of \textit{E. coli} cell lysates with an anti-complex I antibody. By analyzing selected members of our mutant collection, we demonstrated that antibody #2409 recognizes both the NuoCD and NuoG subunits. As predicted, the \(\Delta\textit{nuoG1}\) and \textit{nuoG2} mutants synthesized smaller and larger variants of the NuoG subunit, respectively. Since all fourteen genes appear to be transcribed by \(\Delta\textit{nuoG1}\) and \textit{nuoG2} cells, these cells presumably synthesized wild-type versions of all the other Nuo subunits. Therefore, we conclude that functional complex I requires NuoG.

Fe-S centers were detected in the cytoplasm of the \textit{nuoN} mutant by EPR spectroscopy. The detection of these centers in the cytoplasm instead of associated with the membrane supports the hypothesis that NuoE, NuoF, and NuoG subunits can form the peripheral NDF in the absence of the membrane fragment. Furthermore, Braun and
colleagues (14) have recently shown that the NDF is properly assembled without the membrane fragment as long as the NuoCD subunit of the connecting fragment is present. We demonstrate here that the C-terminal defects caused by deletion or duplication of the 235-bp CTR prevent complex I from functioning properly. Perhaps the altered NuoG subunits cannot properly incorporate into the NDF or, if incorporated, prevent the further assembly of other subunits.

**Expression Studies**

On the basis of the following evidence, we hypothesize that NuoG and at least one other downstream subunit affect *nuo* promoter activity. First, of all the mutants tested, only those carrying the Δ*nuoG1* or the *nuoG2* mutation exhibited promoter activity markedly reduced from that exhibited by wild-type cells when transformed with the multi-copy *nuoPA'::lacZYA* operon fusion. This effect was not observed in *nuoG::Tn10* or *nuoH::Km* transformants suggesting that it is not the lack a functional NuoG alone that causes an inhibitory effect at the *nuo* promoter. Second, this inhibitory effect was alleviated by the additional presence of the *nuoH::Km* or *nuoI::Km* mutations. To a lesser extent, this was also true of the *nuoM::Cm* or *nuoN::Km* mutations. The *nuoG* double mutants that contain either the *nuoH::Km* or *nuoI::Km* mutations exhibited a significant increase in promoter activity compared to those *nuoG* double mutants that contain either the *nuoM::Cm* or *nuoN::Km* mutations. This suggests that the repression observed in our experiments is orchestrated by a Nuo subunit(s) downstream of NuoH, yet upstream of NuoM.
The overexpression of *nuoH-N* experiments are still incomplete. Since we observed the same effect on promoter activity in cells expressing *nuoH-N* in both orientations, we cannot conclude whether the effect is specifically due to the expression of those genes. First, the expression of *nuoH-N* from each construct would need to be verified by transcriptional and/or translational analysis. Second, if *nuoH-N* is being expressed, the origin of expression needs to be determined in the construct where the genes are oriented opposite that of the P_{BAD} promoter. Finally, the gene(s) responsible for the effect would need to be determined through deletion and mutational analysis.

Our data also is consistent with the ability of NuoA to exert influence on *nuo* promoter activity: cells that overexpress *nuoA* exhibited a reduced level of promoter activity relative to those that do not overexpress *nuoA*. Further support for this hypothesis comes from primer extension data from the *S. typhimurium* promoter (3, 29). *E. coli* cells transformed with a multi-copy plasmid containing both the *S. typhimurium* *nuo* promoter and *nuoA* exhibited the same levels of primer extension products as did those transformed with a control vector or untransformed *S. typhimurium* cells. These data are consistent with a role for NuoA as a repressor of *nuo* expression.

The results of the preceding experiments indicate that some of the Nuo subunits may serve dual roles: they can act as structural components that participate in the enzymatic activities of complex I plus they can function in a regulatory capacity. A membrane-associated protein in *S. typhimurium*, PutA, is such a bifunctional enzyme. It both couples the oxidation of proline to glutamate with the transfer of electrons directly to the electron transport chain in the cytoplasmic membrane and acts as a transcriptional
repressor that binds DNA to regulate the put operon in response to proline (85). The two activities are mutually exclusive and are determined in part by the cellular location of PutA (80).

Both the NuoA and NuoH subunits contain putative DNA-binding regulatory domains, consistent with the hypothesis that each possesses regulatory activity. The NuoA subunit contains a LysR-like, helix-turn-helix DNA-binding motif located at its N-terminus, consistent with other members of the LysR family (42, 97). The NuoH subunit contains an AraC-like, helix-turn-helix DNA-binding motif appropriately located at its C-terminus (19, 42). The hypothesis that NuoA can act as a negative regulator is supported empirically by the decrease in promoter activity in cells overexpressing nuoA. The hypothesis that NuoH serves as a regulator is supported by the experiments in which the overexpression of nuoH-N resulted in a decrease in nuo promoter activity.

**Model for complex I Assembly**

The fungus *Neurospora crassa* assembles complete mitochondrial complex I *en bloc* from two independently assembled subcomplexes (25, 31, 34, 57, 81, 98, 100, 108). The peripheral arm, comprised of only nuclear-encoded subunits, was shown to assemble independently of the membrane arm (34); while the membrane arm, comprised of both nuclear- and mitochondrial-encoded subunits, was shown to assemble independently of the peripheral arm (98). Once both arms are made, they are assembled into a single enzyme complex at the mitochondrial matrix. If the NuoG homolog in *N. crassa* (Nuo78) is deleted, then only the membrane arm of complex I is detected, the peripheral
arm is not (41, 54). Similar results have been achieved after deleting other *N. crassa* (25, 31, 57, 81, 100) or human (57) complex I subunits. If a subunit from one arm is mutated, the other arm can still subassemble.

We hypothesize that *E. coli* complex I assembly also proceeds first by constructing independently assembled subcomplexes (Fig. 20). However, since *E. coli* harbors all of its *nuo* components in a single cellular compartment, it is logical that the regulation of expression and assembly would differ somewhat from that of the eukaryotes. This hypothesis is inferred from our mutant data. We did not detect any complex I activity or subcomplex formation in any of our *nuo* mutants, with the exception of the *nuoN* mutant, in which an NDF was detected.

The following evidence supports the hypothesis of *en bloc* assembly of *E. coli* complex I: i) the conserved clustering of the *nuo* locus (38, 68), ii) the evolution of complex I (38-40, 68), iii) the detection of the NDF in the *nuoN* mutant (10), and iv) the observation that the NDF is properly assembled in the absence of the membrane fragment when NuoCD of the connecting fragment is present (14). On the basis of this information, we propose that the Δ*nuoG1* and *nuoG2* mutants cause the complex I defect because the altered NuoG subunits that they synthesize cannot be assembled into the NDF properly. This, in turn, prevents further assembly of other Nuo subunits. This hypothesis is supported by the inability to detect any Fe-S centers, present in the peripheral and connecting fragments, by EPR spectroscopy in the Δ*nuoG1* mutant.
Fig. 20. Model for complex I assembly. The NuoCD subunit of the connecting fragment is necessary for the peripheral subunits NuoE, NuoF, and NuoG to form the peripheral fragment (NDF). Once the NDF and NuoCD have assembled, the remaining connecting fragment subunits (NuoB and NuoI) can assemble. Next, the membrane fragment assembles. It is unclear whether the membrane fragment assembles with the rest of the subassembled complex I as a block or one subunit at a time.
To address the hypothesis that the ΔnuoG1 and nuoG2 mutations result in altered NuoG subunits that cannot assemble into an NDF, a system similar to that used by Braun and colleagues (14) can be employed. They have already established a system whereby they can demonstrate formation of the NDF independent of the membrane fragment when nuoB-G are expressed from an expression vector. In their system, wild-type nuoB-G is cloned into an expression vector. That same system could be used where nuoB-ΔnuoG1 or nuoB-nuoG2 are cloned into the vector instead. Then cells transformed with these expression constructs can be evaluated for their ability to assemble an NDF.

Furthermore, non-polar mutations introduced into nuoB, nuoC, or nuoD in the expression constructs could provide information concerning which of the connecting subunits are necessary for the assembly process. In conjunction with these genetic experiments, pulse-labeling and immunoprecipitation experiments similar to those employed by *N. crassa* complex I researchers (34) can be performed to witness the formation of NDF assembly intermediates.

**Model for the Regulation of nuo Expression**

Perhaps throughout its evolution, the *nuo* locus retained its precursors' regulatory components to coordinate regulation of all its components, ensuring proper expression and assembly of a complete and functional complex I. On the basis of our findings, we hypothesize that *E. coli* cells can sense whether all of the Nuo subunits have been synthesized and assembled, utilizing some form of feedback mechanism to regulate the expression of *nuo* (Fig. 21). This mechanism would allow the cell to shut-down
Fig. 21. Model for the feedback mechanism of regulation of nuo expression. The ΔnuoG1 mutant synthesizes a shortened, defective form of the NuoG subunit. This altered NuoG subunit is unable to assemble as part of the NDF and therefore into the complete complex I. Consequently, the connecting and membrane fragments are also unable to assemble into the complete complex I. As a result, either the membrane fragment and/or the subunits that constitute the membrane fragment are in excess in the cytoplasm and are able to repress transcription from the nuo promoter. Thus, the Nuo subunits participate in the regulation of nuo expression. This mechanism would allow the cell to shut down expression of nuo when a functional complex I can not be made.
expression of *nuo* when a functional complex I could not be made. Feedback mechanisms are used by a number of other large protein complexes in *E. coli*, e.g. the flagellar apparatus (70) and ribosomes (84, 121) to coordinately regulate their expression and assembly.

The data that supports our hypothesis for feedback regulation of *E. coli* complex I is as follows. First, the Δ*nuoG1* and *nuoG2* mutants do not make a functional complex I. Second, these mutants synthesize defective forms of the NuoG subunit but, presumably, synthesize all other Nuo subunits. Third, activity from the *nuo* promoter is reduced i) in the Δ*nuoG1* and *nuoG2* mutants, ii) in cells that overexpress *nuoA*, and iii) in cells that overexpress *nuoH-N*. These data may reflect separate, unrelated aspects of regulation; alternatively, these data may provide insight into the overall regulatory network. Finally, the reduced promoter activity observed in the Δ*nuoG1* and *nuoG2* mutants is repressed by the absence of NuoH-N. As stated above, we hypothesize that the altered NuoG subunits cannot properly incorporate into the NDF. Then, in turn, the NDF would not be available, so some or all of the components of the membrane fragment cannot associate with the connecting fragment. This would cause the membrane subunits or the membrane fragment to be in excess in the cytoplasm. Perhaps NuoA, NuoH, or a complete subassembled membrane fragment could reduce expression of *nuo* by inhibiting transcription at the *nuo* promoter.

The feedback regulation model we present concerning Nuo subunits affecting *nuo* regulation arose from our studies with multi-copy operon and protein fusions. To strengthen the physiological relevance of our results, it would be necessary to move to
single-copy fusion studies. For example, we could further address the impact of NuoA on the nuo promoter by using a single-copy nuoP::lacZYA operon fusion in conjunction with an inducible expression construct expressing nuoA. If NuoA does act as a repressor, then we would expect to observe a reduction in promoter activity from the single-copy fusion when nuoA expression is induced. Additionally, our model implies that one or more Nuo subunits interact directly with the nuo promoter. There are a number of experimental approaches that can be used to determine whether Nuo subunits bind the promoter. First, the subunits of interest (for instance NuoG, NuoA, and NuoH) need to be synthesized and purified. Then a variety of in vitro molecular techniques, such as gel shift analysis and DNase I footprinting studies, can be employed to determine whether those subunits do, in fact, bind to the nuo promoter and, more specifically, where that binding occurs on the DNA. Using antibodies directed against those subunits, the specificity of binding can be addressed.

Sigma Factor Regulation

A great deal of information has been attained concerning external regulators of nuo. Researchers have demonstrated a role for both electron acceptors and electron donors (12, 106) as well as global transcriptional regulators (12) in the regulation of nuo expression. These studies provide insight into the environmental stimuli that affect nuo expression. However, studies concerning which sigma factor(s) is responsible for nuo expression have not been performed. Here, we present the first data concerning that topic.
We measured the β-galactosidase activity in three mutants transformed with the *nuoPA*:::*lacZ* operon fusion, each defective in one of three (69) *E. coli* sigma factors, \(\sigma^{70}\), \(\sigma^{5}\), and \(\sigma^{54}\). We observed that β-galactosidase activity was significantly decreased only in cells defective for \(\sigma^{54}\) (RpoN) but not for either \(\sigma^{70}\) (RpoD) or \(\sigma^{5}\) (RpoS). Contrary to earlier speculation (3, 12, 114), these findings suggest that the major sigma factor of exponential growth, \(\sigma^{70}\), is not likely to be the major sigma factor controlling *nuo* expression. Furthermore, according to our results, \(\sigma^{5}\), the major sigma factor during stationary phase and the response to oxidative stress, also does not appear to be the major sigma factor contributing to *nuo* expression. Instead, our results are consistent with \(\sigma^{54}\), the sigma factor responsible for nitrate and formate metabolism and anaerobic gene expression, serving as the major sigma factor regulating *nuo* expression. While the -10/-35 \(\sigma^{70}\)-binding consensus sequences in any one promoter is not highly conserved (45), \(\sigma^{54}\)-dependent promoters conform to a precise consensus sequence TGGCAC-N5-TTGT/ga/t located between -26 and -11 (76, 79). Surprisingly, we did not find this consensus sequence anywhere within the *nuo* promoter. This suggests that \(\sigma^{54}\) may i) bind to some region of the promoter other than the known consensus sequence or ii) that \(\sigma^{54}\) may work through an intermediate to regulate *nuo* expression, perhaps in conjunction with another sigma factor(s). The most likely candidates are still \(\sigma^{70}\) and \(\sigma^{5}\). One hypothesis is that both \(\sigma^{70}\) and \(\sigma^{5}\) initiate transcription from the *nuo* promoter, but only in the presence of some factor, factor X, expressed by \(\sigma^{54}\). If either \(\sigma^{70}\) or \(\sigma^{5}\) are absent, the system does not get shut-down. However, if \(\sigma^{54}\) is absent, then factor X cannot be synthesized and neither \(\sigma^{70}\) nor \(\sigma^{5}\) can function.
It is clear from our data that the role of sigma factors in the regulation of *nuo* expression should be analyzed further. There are no studies addressing the role of sigma factors in the regulation of any of the components of the respiratory chains (47). Our results establish a role, direct or indirect, for $\sigma^{54}$ in the expression of *nuo* yet do not completely rule out a role for either $\sigma^{70}$ or $\sigma^{5}$. It is necessary to determine which of the sigma factors bind the *nuo* promoter. DNaseI footprinting and gel shift analyses have proven to be reliable techniques for addressing this question (15, 49). To further define the sigma factor/promoter interactions, *in vitro* transcription with reconstituted holoenzyme also can be employed (105). Of course, it is possible that the $\sigma^{54}$ effect we observed is indirect. Then, it would be necessary to identify what intermediate(s) through which $\sigma^{54}$ functions. This might be accomplished using an *E. coli* library in conjunction with the *nuoPA'*::*lacZYA* operon fusion to identify genes that affect *nuo* transcription through $\sigma^{54}$.

**Long-term Goal**

Thinking further into the future, a long-term goal may be to address how the mechanism of *nuo* regulation works in concert with that of other respiratory chain elements to balance a cell's redox state and to maximize its energy production. We already understand to some degree, the environmental factors that regulate expression of the respiratory chain enzymes. A more in depth examination of the coordinated mechanisms will provide a great deal of insight into how energy metabolism is regulated in the cell.
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The author, Holly J. Falk-Krzesinski, was born in Skokie, Illinois to Ileen and Kenneth Falk. She received her secondary education at Maine Township High School East in Park Ridge, Illinois where she graduated in 1988. She then attended the University of Illinois at Chicago where she received a Bachelor of Liberal Arts and Sciences degree with college honors in 1992. During this time, Ms. Falk-Krzesinski pursued a Biological Sciences major and a minor in Chemistry as well as performed an undergraduate research project in the laboratory of Dr. F. Marion Hulett.

During her studies at UIC, Ms. Falk-Krzesinski was employed by Abbott Laboratories as a research intern in the Pharmaceutical Products Research and Development Division under the guidance of Dr. Robert Simmer. She was involved in the expression and purification of proteins with pharmaceutical potential.

In August 1992, Ms. Falk-Krzesinski began her graduate studies in the Department of Microbiology and Immunology at Loyola University Chicago. The following August, she married Mitch Krzesinski. One month later, she joined the laboratory of Dr. Alan J. Wolfe. In Dr. Wolfe's laboratory, Ms. Falk-Krzesinski focused her efforts on studying how *Escherichia coli* cells regulate the expression and assembly of the respiratory chain enzyme, complex I.
Ms. Falk-Krzesinski was the recipient of a number of fellowships and travel grants throughout her graduate career. These include the Association for Women in Science Educational Foundation Amy Lutz Rechel Foundation Award in 1997, the American Society of Microbiology/American Academy of Microbiology Raymond W. Sarber Award in 1997, the Sigma Delta Epsilon/Graduate Women in Science Eloise Gerry Research Fellowship in 1996, the Alpha Kappa Alpha Educational Advancement Foundation Alice Thomas Motts Trust Merit Scholarship in 1996, and the Loyola University Dissertation Fellowship in 1996.

Ms. Falk-Krzesinski has accepted a position as a Postdoctoral Research Associate in the laboratory of Dr. Gail Hecht in the Department of Medicine, Section of Digestive and Liver Disorders at the University of Illinois at Chicago. There she will investigate the effects of enteropathogenic *Escherichia coli* on the host immune response at the molecular level.
The dissertation submitted by Holly J. Falk-Krzesinski has been read and approved by the following committee:

Alan J. Wolfe, Ph.D., Advisor
Associate Professor, Microbiology and Immunology
Loyola University Chicago

Adam Driks, Ph.D.
Assistant Professor, Microbiology and Immunology
Loyola University Chicago

David Hecht, M.D.
Associate Professor, Infectious Diseases
Loyola University Chicago

Robert Gennis, Ph.D.
Professor, Biochemistry
University of Illinois, Champaign-Urbana

F. Marion Hulett, Ph.D.
Professor, Laboratory for Molecular Biology
University of Illinois, Chicago

The final copies have been examined by the advisor of the dissertation and the signature which appears below verifies the fact that any necessary changes have been incorporated and that the dissertation is now given final approval by the committee with reference to content and form.

The dissertation is, therefore, accepted in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

11/24/97
Date

[Signature]
Advisor's Signature