

COMMUNICATION

Genetic Evidence for the Interaction between Cluster I and Cluster III Rifampicin Resistant Mutations

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Rifampicin-resistant (Rif<sup>r</sup>) mutations of *Escherichia coli* map to the central portion of the *rpoB* gene, which encodes the  $\beta$  subunit of RNA polymerase. These mutations are located in three distinct clusters, designated I, II and III. Three intragenic suppressors of the cluster III Rif<sup>r</sup> mutation, *rpoB3406*(RH687), restore the ability of the mutant strain to grow at low and high temperatures and map to a single locus in cluster I. These suppressors are identical to two previously characterized Rif<sup>r</sup> alleles, *rpoB3401*(RC529) and *rpoB3402*(RS529). None of the other 14 previously identified Rif<sup>r</sup> mutations that we have characterized confers this phenotype. We suggest that this allele-specific suppression results from interaction between Cluster I and Cluster III of the  $\beta$  subunit.

**Keywords:** RNA polymerase; Rif<sup>r</sup> mutants; termination; antitermination

*Escherichia coli* RNA polymerase consists of four core subunits ( $\alpha_2\beta\beta'$ ) required for transcript elongation and termination and an additional subunit, called sigma, required for specific initiation at promoter regions of the DNA (for a review, see Burgess *et al.*, 1987). The  $\beta$  subunit of RNA polymerase, a polypeptide of 1342 amino acid residues (Ovchinnikov *et al.*, 1981, 1982) is likely to be part of the catalytic center of the enzyme (Grachev *et al.*, 1987, 1989; Jin & Gross, 1991; Jin *et al.*, 1992; Kashlev *et al.*, 1990; Lee *et al.*, 1991). An understanding of the structural organization of this subunit is thus of great interest. Moreover, because the  $\beta$  subunit is homologous in several regions to the second largest subunit of eukaryotic RNA polymerases (Sweetser *et al.*, 1987; Falkenburg *et al.*, 1987; Allison *et al.*, 1985; Biggs *et al.*, 1985; Ovchinnikov *et al.*, 1981, 1982) a structure-function analysis of this subunit will be relevant to eukaryotic polymerases as well as to their prokaryotic counterparts.

Very little detailed structural information exists either about RNA polymerase or the  $\beta$  subunit. The size and complexity of the enzyme has precluded detailed physical studies, and the fact that it is an

essential enzyme has made genetic analysis very difficult. Nonetheless, some information is available. A low resolution map of *E. coli* RNA polymerase has been obtained from electron crystallography studies on two-dimensional RNA polymerase crystals (Darst *et al.*, 1989). In addition, the nucleotide binding site in the  $\beta$  subunit has been partially defined. Affinity cross-linking studies indicate that amino acid residues located around positions 550, 1036 to 1066 and 1234 to 1242 of the  $\beta$  polypeptide are in the immediate vicinity of the initiating nucleotide (Grachev *et al.*, 1989; Grachev, personal communication). Genetic evidence supports the idea that amino acid residues around 550 participate in nucleotide binding: a mutational change at Arg529 confers resistance to rifampicin and also alters the binding of purine nucleotides to the elongating transcript (Jin & Gross, 1991).

We are particularly interested in understanding the structure of the region of the  $\beta$  subunit where mutational changes can lead to resistance to rifampicin (Rif<sup>r</sup>). The phenotypes of Rif<sup>r</sup> mutations indicate that this region of RNA polymerase plays a central role in the function of the enzyme. In addition to the implied association with the nucleotide binding site mentioned above, Rif<sup>r</sup> mutations lead to altered termination and antitermination phenotypes (Jin *et al.*, 1988a,b), suggesting that this region of RNA polymerase also participates in these processes. Rif<sup>r</sup> mutations are located in three clusters in the middle of the *rpoB* gene encoding the

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**Table 1**  
*Strains, phages and plasmids used in this study*

Name	Relevant genotype or construction	Source or reference
MG1655	Wild-type	B. Bachmann
MG1655 <i>rpoB3401</i>	<i>rpoB3401</i> (RC529)†	Jin & Gross (1988)
MG1655 <i>rpoB3402</i>	<i>rpoB3402</i> (RS529)†	Jin & Gross (1988)
MG1655 <i>rpoB3406</i>	<i>rpoB3406</i> (RH687)†	Jin & Gross (1988)
MG1655 <i>rpoB3401, 3406</i>	<i>rpoB3401, 3406</i> (RC529, RH687)†	This work
MG1655 <i>rpoB3402, 3406</i>	<i>rpoB3402, 3406</i> (RC529, RH687)†	This work
CAG8102	K37 <i>galK2, rpsL<sup>+</sup></i> derivative	Jin & Gross (1988)
CAG8333	SA500 <i>musA1 his ilv galE490</i> ( <i>chlD-blu</i> ) <sup>AB</sup> ( $\lambda$ DBAM <i>N<sup>+</sup> cI14 <math>\Delta</math>H) Gal operon under control of <math>\lambda P_L</math></i>	Jin <i>et al.</i> (1988a)
$\lambda$ chiron 25	Lysogenic $\lambda i^{21}$ cloning vector	Gross <i>et al.</i> (1979)
pRZ150	Amp <sup>r</sup> , contains M13 origin of replication	W. Reznikoff
pD12	No terminator	Luk & Szybalski (1982)
pD129	$\lambda t_{R1}$ Rho-dependent terminator	Luk & Szybalski (1982)
pD34	$\lambda t_{L2a}$ Rho-independent terminator	Luk & Szybalski (1983)
pDS367	$\lambda t_{L2b}$ Rho-independent terminator	Luk & Szybalski (1983)
pDJJ11-3406	pDJJ11 containing the <i>rpoB3406</i> allele	Jin & Gross (1988)

† Amino acid changes are in parentheses.

$\beta$  subunit Jin & Gross (1988). More than 90% of the Rif<sup>r</sup> mutations are located in Cluster I, encompassing amino acid residues 505 to 532 and most of the remainder are located in Cluster II, comprising amino acid residues 560 to 572. A single Rif<sup>r</sup> mutation, located at amino acid 687 defines Cluster III. This report centers on understanding the relationship of the Cluster III Rif<sup>r</sup> mutation, *rpoB3406*(RH687), to the rest of the Rif region (Jin & Gross, 1988). In contrast to other Rif<sup>r</sup> mutations, *rpoB3406* confers resistance only to very low levels of rifampicin (approximately 40  $\mu$ g/ml), which suggests that it might be located at the periphery of the region of RNA polymerase binding rifampicin. In this case, it might be in close proximity to the other Rif<sup>r</sup> mutations in the enzyme. We have isolated and characterized intragenic second site suppressors of the cluster III Rif<sup>r</sup> mutation to investigate this relationship.

#### (a) Isolation of intragenic suppressors of *rpoB3406*

We took advantage of the cold-sensitive (Cs<sup>†</sup>) and thermo-sensitive (Ts) growth phenotypes of *rpoB3406* (see Table 1) to isolate second site suppressors restoring function to the enzyme. Spontaneous cold-resistant (Cr) or thermo-resistant (Tr) suppressors were isolated by plating *rpoB3406* at 20°C or 44.5°C. A low concentration of rifampicin (40  $\mu$ g/ml) was included in the selective plates to maintain the presence of the original Rif<sup>r</sup> mutation. Note that second site suppressors that eliminate Rif<sup>r</sup> will not be obtained in this selection. Tr suppressors occurred at a frequency too high to represent intragenic revertants (approx.  $1 \times 10^{-4}$ ; see Table 2). Cr suppressors, on the other hand,

occurred at a frequency consistent with that expected for intragenic suppression (approx.  $6 \times 10^{-7}$ ) and were further characterized. The seven Cr suppressors were also able to grow at high temperature, indicating that their mutational change restored function of the enzyme at all temperatures. Half of the suppressor strains (3 independent isolates; termed Class I suppressors) were resistant to high levels of rifampicin. The remainder, Class II suppressors, exhibited the low resistance level of the parental strain. Both classes of suppressors were closely linked to *rpoB* by P1 transduction, indicating that they were either in, or very near, this locus (data not shown). Characterization of the Class I suppressors forms the basis of this report.

The Class I suppressors confer resistance to the high levels of rifampicin characteristic of Rif<sup>r</sup> mutations located in Clusters I and II and preliminary mapping experiments indicated that this was their location (M.S., unpublished results). To directly determine the molecular nature of the Class I suppressors, we cloned a 12 kb *Hind*III fragment carrying the *rpoB* gene from each of the suppressors into the Ch25  $\lambda$  vector, subcloned the 238 bp *Bcl*I fragment spanning the region encoding Clusters I and II into pRZ150 and sequenced this fragment with a dideoxy protocol. Sequence analysis indicated that all three suppressors changed base-pair (bp) 1585 relative to the start of the coding region of the gene taken as +1. Two different suppressors were identified, each of which had previously been isolated as single mutations, *rpoB3401*(RC529) and *rpoB3402*(RS529) conferring resistance to high levels of rifampicin and Ts growth (Jin & Gross, 1988). The location of the three single and two double mutations and their corresponding growth phenotypes are summarized in Table 2. To determine if the other suppressor mutations were also located in this region but simply did not confer high level Rif<sup>r</sup>, the *Bcl*I region of the Class II

† Abbreviations used: Cs, cold-sensitive; Ts, thermo-sensitive; Cr, cold-resistant; Tr, thermo-resistant; bp, base-pair(s).

**Table 2**  
Growth phenotypes of single and double  
*rpoB* mutants

<i>rpoB</i> allele	Amino acid affected	EOP† at 20°C	EOP† at 44.5°C
Wild-type		1	1
3406	RH687	$5.9 \times 10^{-7}$	$\sim 1 \times 10^{-4}$
3401	RC529	0.8	$2.2 \times 10^{-6}$
3402	RS529	1	$3.8 \times 10^{-6}$
3401, 3406	RC529, RH687	1	1
3402, 3406	RS529, RH687	1	1

Saturated LB broth cultures grown 30°C were plated for single colonies on LB plates incubated at 20°C, 37°C and 42°C.

† EOP (efficiency of plating) is defined as:

$$\frac{\text{no. of colony forming units at 20°C or 44.5°C}}{\text{no. of colony forming units at 37°C}}$$

suppressors was also sequenced. None of the Class II suppressors were located in the region of RNA polymerase.

To confirm that the suppressed phenotypes were the result of the introduction of the *rpoB3401* and *rpoB3402* mutations, a reconstruction experiment was performed. Plasmids carrying the *BclI* region from the mutant or wild-type strains were introduced into *rpoB3406* and plated at 20°C. *rpoB3406* transformants containing the *BclI* region from *rpoB3401* and *rpoB3402* gave rise to Cr colonies at a frequency at least tenfold greater than those with the wild-type *BclI* region. The Cr colonies were also resistant to high levels of rifampicin and able to grow at high temperature. Thus, a recombinational event introducing the mutation on the *BclI* fragment was sufficient to reconstruct the suppressed phenotype.

The fact that both intragenic suppressors affected a single amino acid suggested that suppression was allele specific, and not a general characteristic of Rif<sup>r</sup> mutations. If so, the other sequenced Rif<sup>r</sup> alleles in our collection should not be able to suppress the growth defects of *rpoB3406*. To determine if this were the case, we asked whether we could detect recombinants between each of the other Rif<sup>r</sup> alleles and *rpoB3406* that suppressed the growth defects of the single mutant parents. We used two different protocols for the experiment. Of the Rif<sup>r</sup> alleles, 11 have either Ts or Cs growth phenotypes. For these alleles, we determined whether introduction of the *rpoB3406* mutation would restore growth at non-permissive temperature. These strains were transformed with a plasmid carrying the *BclI-EcoRI* fragment of *rpoB3406* that covers Cluster III, but not the remainder of the Rif region and plated at non-permissive temperature. Of these 11 strains, only the *rpoB3401* and *rpoB3402* mutant cells gave rise to recombinants that could grow at the non-permissive temperature. We performed a reciprocal experiment for the five Rif<sup>r</sup> alleles that did not have a growth phenotype. We transformed *rpoB3406* with plasmids containing the *BclI* region from each of the mutant *rpoB* genes

**Table 3**  
Relative read-through of single and  
double Rif<sup>r</sup> mutants

<i>rpoB</i> allele	Amino acid affected	Terminators		
		$\lambda t_{L2a}$ †	$\lambda t_{L2b}$ †	$\lambda t_{R1}$ ‡
Wild-type		1	1	1
3406	RH687	1	1	6.2
3401	RC529	1	1.1	7.5
3402	RS529	0.8	0.90	2.6
3401, 3406	RC529, RH687	0.84	0.93*	0.2
3402, 3406	RS529, RH687	0.96	0.63	1.0

Relative read-through is % read-through at a particular terminator normalized to % read-through at the same terminator in the wild-type strain, corrected for copy number of the terminator probe plasmids as described previously (Jin *et al.*, 1988b). The terminator probe vectors carry the galactokinase (GalK) gene located downstream from the indicated terminator (Luk & Szybalski, 1982, 1983). Thus, GalK activity is a measure of extent of termination. GalK activity was assayed from cells growing exponentially in M9 glucose medium supplemented with all amino acids and vitamins. All assays are averages of at least 2 experiments (error is  $\pm 5\%$ ), except where indicated by \* where the assay was performed once. The GalK units in the wild-type strain were 33.6 (control vector, no terminator), 2.16 ( $\lambda t_{R1}$ ), 5.7 ( $\lambda t_{L2a}$ ) and 14.4 ( $\lambda t_{L2b}$ ).

†  $\lambda t_{L2a}$  and  $\lambda t_{L2b}$  are Rho-independent terminators.

‡  $\lambda t_{R1}$  is a Rho-dependent terminator.

(this fragment covers Rif Clusters I and II but not III) and determined whether they could rescue the growth phenotype of *rpoB3406*. None of these strains gave increased frequency of Cr colonies. However, each of these plasmids gave rise to Rif<sup>r</sup> recombinants in a wild-type strain at a frequency between  $8.4 \times 10^{-6}$  and  $1.1 \times 10^{-5}$ . This frequency of recombination is at least tenfold above the reversion frequency of *rpoB3406*. If these recombinants gave rise to Tr growth in *rpoB3406*, they would have been detected. Thus, these Rif<sup>r</sup> alleles are not intragenic suppressors of *rpoB3406*. In conclusion, among our current collection of Rif<sup>r</sup> alleles, only the two Rif<sup>r</sup> alleles that were originally isolated as *rpoB3406* suppressors confer this phenotype.

#### (b) Characterization of the phenotypes of suppressor strains

To characterize the functional state of RNA polymerase in the suppressed strain, we compared the *in vivo* termination and antitermination phenotypes of the double mutants with the single mutant strains. At the Rho-dependent terminator,  $\lambda t_{R1}$ , both of the double mutant strains suppressed the termination defect of the single mutant and restored termination either to the wild-type level (*rpoB3406 3402*) or below (*rpoB3406 3401*) (see Table 3). The enhanced termination phenotype was manifest most strongly at Rho-dependent terminators. Termination at the two Rho-independent terminators examined was affected little or not at all by the double mutant strains (Table 3). We also assayed the effect of the double mutant strain on its ability to suppress the

**Table 4**  
Relative read-through of single and double *Rif<sup>r</sup>* mutants

<i>rpoB</i> allele	Expression of GalK $\lambda P_L$ CAG 8333 (N5261 <i>nusA1</i> )†		
	32°C	38°C	42°C
Wild-type	1.0	1.0	1.0
3406	1.8	2.3	5.1
3401	1.8	5.8	n.d.‡
3402	0.8	2.2	3.4
3401, 3406	1.5	2.5	4.8
3402, 3406	1.2	2.7	3.9

† The amount of galactokinase in each of the mutant strains is presented relative to that in the wild-type strain. In this strain, expression of GalK is under control of the  $\lambda P_L$  promoter and the amount of GalK measures the antitermination activity of N in the *nusA1* mutant background. The GalK units in the wild-type strain were: 20 (32°C), 5 (38°C) and <0.5 (42°C). All assays are averages of at least 2 independent experiments and were within 5% error.

‡ n.d., not determined.

defect in  $\lambda$  N-mediated antitermination exhibited by *nusA1* strains at high temperature. The double mutant retained this phenotype, although the extent of suppression is lower than the single mutant strains and is summarized in Table 4.

The Class I suppressor mutations may function by restoring appropriate interactions between two amino acid residues in close proximity to each other in the protein. Alternatively, they may create compensating functional changes in the enzyme. Although our experiments cannot rigorously distinguish between these two alternatives, our data on function, allele specificity and location of the suppressors are most consistent with the first hypothesis. All of the three single mutant strains have very similar phenotypes: they are Ts, they suppress *nusA1* and most significantly, they are the only *Rif<sup>r</sup>* mutations that decrease termination at Rho-dependent but not Rho-independent terminators. The similarity of the single mutations makes it unlikely that they are suppressing by introducing a compensating functional alteration and suggests an altered structural interaction as a basis for the suppression. This is particularly evident in examining the termination phenotype at  $\lambda t_{R1}$ . Although the single mutants show between three- to sevenfold read through at this terminator, the double mutant strains exhibit either normal termination (*rpoB3402*, *rpoB3406*) or enhanced termination (*rpoB3401*, *rpoB3406*). The fact that all Class I suppressors affect Arg529 also suggests a structural basis for the suppression. If Arg529 and Arg687 were in close proximity in the protein, mutational alterations in either of them might be expected to give similar phenotypes. Changing both arginine residues to other amino acids might allow a more wild-type structure in this region of RNA polymerase than is possible when only one of the arginine residues is altered.

We argue that our suppression data strongly suggest that two discrete regions of RNA polymerase, one located around Arg529 and the second located around Arg687, are in close proximity in the native protein. The close proximity of Cluster I *Rif<sup>r</sup>* mutations at Arg529 with the Cluster III *Rif<sup>r</sup>* mutations at Arg687 suggest that these two regions of the polypeptide are both involved in forming the binding site for rifampicin. These results represent the first evidence for a potential interaction between two amino acid residues in the  $\beta$  subunit of RNA polymerase.

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