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RNA Polymerase Mutants of *Escherichia coli*

II. Streptolydigin Resistance and Its Relation to Rifampicin Resistance

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Summary. Several streptolydigin-resistant mutants of *Escherichia coli* were shown to produce RNA polymerase with increased drug resistance due to a recessive mutation *(stl)* located between *argH* and *thiA*. With one mutant studied, enzyme reconstitution experiments directly demonstrated that the altered β subunit is responsible for its drug resistance. It was also found that some mutations (*rif* or *stv*) conferring resistance to rifampicin (or streptovaricin) lead to a simultaneous change in resistance to streptolydigin, suggesting certain functional relationship between the polymerase structure affected by *rif* (or *stv*) and *stl* mutations. This inference was further supported by the results of cistron analysis and of extensive transductional mapping involving two *stl* and a number of *rif* mutations. Thus it was found that all the mutational sites affecting sensitivity of RNA polymerase to streptolydigin and to rifampicin are closely localized, with partial overlap to each other, within a short segment of the cistron which determines the structure of β subunit. These results led us to propose that the β subunit is directly responsible for catalyzing both initiation and chain elongation steps of RNA synthesis. The possible bearing of the present findings on the structure-function relationship of the polymerase is discussed.

Introduction

Genetic analyses of bacterial RNA polymerase have been carried out in several laboratories since the discovery of a class of antibiotics that inhibit RNA synthesis by direct interaction with the enzyme. Thus most mutants of *Escherichia coli* resistant to rifamycins (including rifampicin) or streptovaricins which inhibit initiation of RNA synthesis were found to produce the drug-resistant RNA polymerase due to a mutation (*rif* or *stv*) mapped between *argH* and *thiA* (77.3 to 77.8 min on the Taylor's map) (see reviews by Burgess, 1971; Sethi, 1971). The mutant polymerases have been shown to be defective in their ability to bind rifampicin (Wehrli *et al.*, 1968; Wehrli and Staehelin, 1971; Ishihama and Yura, unpublished results). These results as well as the direct demonstration of the altered β subunit in the polymerase produced by one of the rifampicin-resistant mutants (Heil and Zillig, 1970) establishes that at least the structural gene for β subunit is located on this region of the chromosome.

On the other hand, streptolydigin is known to inhibit the elongation of RNA chains (Siddhikol *et al.*, 1969), and analysis of RNA polymerase resistant to this drug was expected to identify the subunit participating in this reaction. Heil and Zillig (1970) recently obtained the data indicating the alteration of β subunit in a streptolydigin-resistant (*stl*) mutant originally isolated by Schleif (1969). The genetic mapping data reported by Sokolova *et al.*, (1970) suggested, however, that the *stl* mutation they examined might have occurred at the gene distinct

from that for the *rif* mutation. In order to identify and characterize the functional sites on the polymerase that are responsible for the initiation and elongation steps of RNA synthesis, it appeared highly desirable to obtain further information on the genetic and functional relationships between *rif* (or *stv*) and *stl* mutations.

In this report, it will be shown that the β subunit of RNA polymerase is altered in an *stl* mutant as examined by the enzyme reconstitution techniques developed in our laboratory. Taken together with the results of cistron analysis and extensive transductional mapping experiments, it has been concluded that all the *stl* and *rif* mutations tested affect one and the same cistron determining the structure of the β subunit of RNA polymerase.

Materials and Methods

Bacterial Strains. All bacterial strains used in this study are derivatives of *Escherichia coli* K12 and are listed in Table 1.

Table 1. Bacterial strains

Strain	Genetic characters	Origin or source
KY1330	F$^-$ *argE, ppc, metB, his, recA, str, supE* (Stl permeable)	KY1326
KY1340	F$^-$ *argE, metB, trp, tyr, his, recA* (Stl permeable)	
KY1342	HfrH *met, thiA* (Stl permeable)	KY118
W1655recA/KLF10	F′ *metB, recA*/KLF10	S. J. Austin
AB1206	F′ *pro, his, gal, lac, str*, deletion (*ilv-argH*)/F14	E. A. Adelberg
W3949	F$^-$ *argH, gal, str*	S. Udaka
AB313	Hfr *thr, leu, thi, lac, str*	N. Otsuji
2016	Hfr *purD*	N. Glansdorff

Media. PG medium contained 20 g of polypeptone (Wako Drug Co.), 2 g of glucose and 5 g of NaCl per liter, and the pH was adjusted to 7.2. Medium E (Vogel and Bonner, 1956) with appropriate supplements of amino acids (20 μg/ml) or thiamine (2 μg/ml) was used as a standard minimal medium.

Chemicals and Antibiotics. Unlabeled ribonucleoside triphosphates were obtained from Sigma Chemical Company, U.S.A., and C. F. Boehringer and Soehne, Mannheim, Germany. ^{3}H-ATP and ^{3}H-UTP were products of Schwarz Bioresearch Inc., U.S.A. Sephadex G-200, DEAE-Sephadex A-50, and Sepharose 6-B were purchased from Pharmacia, Sweden. Cellulose acetate sheet was a product of Gelman Instrument Company, Germany. Solution of recrystallized urea was freshly prepared before use and deionized by passing through columns of the mixed resin of Amberlite IRC-50 and IRA-400, and Bio-Rad AG 501-X8, 20 to 50 mesh. Streptolydigin (free acid) and streptovaricins (streptovaricin complex) were kindly supplied by Dr. G. B. Whitfield, Jr. of the Upjohn Company, U.S.A. Rifampicin was a product of Lepetit S.P.A., Italy and was generously supplied by Dai-ichi Seiyaku Company, Japan.

RNA Polymerase. Crude extracts were used in most experiments determining resistance or sensitivity of RNA polymerase to the antibiotics. For preparation of crude extracts, cultures (30 ml) were grown to the late log phase at 37° C. Cells were harvested by centri-

fugation, washed and resuspended in 1 ml of buffer A (Chamberlin and Berg, 1962) and disrupted by an ultrasonic oscillator (Kaijo Denki, 29kc). Extracts were spun at 8000×g for 15 min and the resulting supernatants were used as crude extracts.

For the reconstitution experiments, RNA polymerase was purified according to the procedure of Ishihama (1972) with the following modifications; Gel filtration through Sepharose 6-B in the presence of 0.05 M KCl was inserted before DEAE-Sephadex A-50 column chromatography. The enzyme preparations thus obtained contained no more than 3% of contaminants as analyzed by polyacrylamide gel electrophoresis in the presence of SDS.

The reaction mixture (0.25 ml) contained the following in μmoles: Tris-HCl buffer (pH 7.8 at 37° C), 30; $MgCl_2$, 1.25; $MnCl_2$, 0.5; phosphoenolpyruvate, 0.5; pyruvate kinase, 5 μg; ATP, GTP, UTP, CTP, 0.04 each; ^{3}H-ATP (or ^{3}H-UTP), 0.04 μc/mμmole; DNA and enzyme as indicated for each experiment. No exogenous DNA was added when crude extracts were used.

Isolation of Streptolydigin-Resistant Mutants. E. coli strains permeable to streptolydigin were obtained according to Schleif (1969) and were used for isolation of streptolydigin-resistant RNA polymerase mutants. Mutagenized cells were grown overnight, treated with 1 mM EDTA-10 mM Tris-HCl (pH 8.2) containing 100 μg per ml of streptolydigin at 37° C for 20 min, and were plated on PG agar containing the same amount of the drug. The resistant strains thus obtained were tested for drug resistance of RNA polymerase in the *in vitro* system for RNA synthesis.

Transduction with Phage P1. Phage P1-mediated transduction was performed according to the procedure of Lennox (1955). Since strain KY 1330 and its derivatives supported little growth of phage P1, they were outcrossed to an Hfr strain AB313 to isolate *recA*$^+$ recombinants that are fully susceptible to P1 (KY 1344 and KY 1347). When the recipient strain used carried a drug-resistant marker, recombinants were first purified by single colony isolation on the selective agar, and the distribution of unselected markers was determined. When the recipient strain was sensitive to both drugs, recombinant colonies were picked, patches were made on the same selective agar, and the latter was replicated onto appropriate medium to score the unselected markers. Both *rif* and *stl* markers were scored on PG medium containing 100 μg per ml of rifampicin or streptolydigin; 0.25% yeast extract was also added in some experiments.

Reconstitution of RNA Polymerase from Isolated Subunits. Reconstitution of RNA polymerase was carried out as described by Ishihama and Ito (1972), except that subunits were prepared by one of the two following procedures to minimize irreversible inactivation of subunits in 6 M urea solution: the β' subunit and a mixture of the α and β subunits were obtained by column chromatography on DEAE-Sephadex A-50, whereas the pure α subunit and a mixture of the β' and β subunits were obtained by gel filtration through Sephadex G-200. Reconstitution of active enzyme was carried out by mixing the isolated subunit fractions in the stoichiometric ratio and dialyzing against the reconstitution buffer for about 12 hr at 4° C.

Results

Isolation of Streptolydigin-Resistant Mutants

Escherichia coli is usually insensitive to streptolydigin due to the impermeability to the drug. Strains KY 1330 and KY 1331 that are permeable and thus sensitive to the drug (at the concentration of 100 or 20 μg/ml, respectively) were used for isolation of resistant mutants. After mutagenesis of these permeable strains with N-methyl-N'-nitro-N-nitrosoguanidine, 2-aminopurine or nitrous acid, 101 streptolydigin-resistant mutants were isolated independently. As reported previously by other workers, most of the mutants produced streptolydigin-sensitive RNA polymerase indistinguishable from the wild-type enzyme. Only four (designated *stl-1, 4, 5,* and *7*) out of sixty-five resistant mutants obtained after nitrosoguanidine treatment and one *(stl-6)* of the seven mutants obtained by nitrous acid treatment were found to be resistant at the level of

Table 2. Streptolydigin resistance of haploid and merodiploid strains *in vivo* and *in vitro*

Strain	*stl* marker on		Stl phenotype	Residual polymerase activity (%)	
	Chromosome	Episome (KLF 10)		Stl (20 μg/ml)	Stl (100 μg/ml)
KY1330	*stl*+	—	Sensitive	26	7
	stl+	*stl*+	Sensitive	24	8
KY1343	*stl-1*	—	Resistant	75	39
	stl-1	*stl*+	Sensitive	43	19
KY1345	*stl-4*	—	Resistant	77	40
	stl-4	*stl*+	Sensitive	—	—
KY1360	*stl-5*	—	Resistant	74	34
	stl-5	*stl*+	Sensitive	40	14
KY1361	*stl-6*	—	Resistant	54	15
	stl-6	*stl*+	Sensitive	43	11
KY1348	*stl-7*	—	Resistant	86	36
	stl-7	*stl*+	Sensitive	38	13

Cultures were grown at 37° C in medium E supplemented with arginine, glutamate, methionine and histidine (for haploid strains) to the late log phase. For merodiploid strains, arginine, glutamate and methionine were omitted to prevent growth of possible F^- segregants. Stl phenotype was determined by streak test on PG medium containing 100 μg/ml streptolydigin. Crude extracts were used for *in vitro* experiments. Streptolydigin was added to crude extracts before addition of other components of the reaction mixture.

RNA polymerase. No such mutants were obtained spontaneously or after mutagenesis with 2-aminopurine. As can be seen in Table 2, RNA synthesis catalyzed by crude extracts from these mutants were 10 to 20 times more resistant to the drug as compared to the wild-type extract. A quantitatively similar result was obtained with purified RNA polymerase from one of the mutants, as will be shown below (Fig. 3). Thus, these mutants appeared to produce RNA polymerase with altered resistance to streptolydigin.

All these mutations seem to be located on the chromosomal segment covered by the F′ episome F14 or KLF10, since merodiploids carrying either episome were invariably found to be sensitive to streptolydigin (100 μg/ml). This was also confirmed at the level of RNA polymerase. The results with merodiploids carrying KLF10 are presented in Table 2. The partial resistance of RNA polymerase from merodiploids suggests the presence of both streptolydigin sensitive and resistant enzymes in these strains, although the cell growth is inhibited by the drug under the conditions used, indicating that the resistance is recessive to the sensitivity, as has also been reported by Ilyina *et al.* (1971).

Effect of rif (or stv) Mutation on the Sensitivity to Streptolydigin

In the course of studies on rifampicin-resistant mutants obtained from a streptolydigin-permeable strain (KY 1342), it was noted that some of them became more resistant to streptolydigin. Furthermore, streptolydigin sensitivity

Table 3. Rifampicin (or streptovaricin) resistance mutations affecting streptolydigin resistance

Parental strain		Mutants selected	Numbers tested	Change in Stl phenotype		
No.	Stl phenotype			None	Increased resistance	Increased sensitivity
KY1342 (*stl*$^+$)	S	Rif-R	50	40	10	—
KY1340 (*stl*$^+$)	S	Stv-R	11	8	0	3
KY1345 (*stl-4*)	R	Rif-R	20	16	2	2
KY1348 (*stl-7*)	R	Rif-R	7	5	0	2

Rifampicin (or streptovaricin)-resistant mutants were obtained after treatment with ethylmethane sulfonate or 2-aminopurine. Streptolydigin sensitivity was checked by streak tests on PG medium containing 100 μg/ml streptolydigin.

in those mutants appeared to be due to the resistance of RNA polymerase itself rather than to the altered permeability. Thus a number of mutants resistant to rifampicin and streptovaricin were isolated from streptolydigin-sensitive and resistant strains, and simultaneous changes in sensitivity to streptolydigin were examined. As shown in Table 3, a considerable fraction of the mutants obtained independently from several strains are altered in their sensitivities to streptolydigin, though the alterations were to either direction: some became more resistant whereas others more sensitive. It may be noted that not only single *rif* (or *stv*) mutations can lead to simultaneous changes in sensitivities to both rifampicin and streptolydigin, but a *rif* mutation added as the second mutation to *stl* mutants can also affect the sensitivity to streptolydigin.

In agreement with these results, RNA synthesis catalyzed by RNA polymerase (crude extract) from these mutants also exhibited alterations in streptolydigin sensitivity in addition to the rifampicin (or streptovaricin) resistance. Some of the typical results are shown in Fig. 1. Although the extents of alteration were relatively small, the results are quite reproducible and coincided well with the data *in vivo*. These results strongly suggest that the *rif* (or *stv*) and *stl* mutations affect the same cistron or two different cistrons whose products are functionally closely related to each other.

Cistron Analysis between stl and rif Mutations

In order to find out whether the cistron affected by the *stl* mutations is the same or different from that involved in the *rif* mutations, the F′ episomes carrying a *rif0* mutation (Austin and Scaife, 1970) were isolated and transferred into the F$^-$ streptolydigin-resistant mutants to obtain merodiploids with the genotype, *stl*/KLF10 *rif0*. The *rif0* mutations presumably inactivate the *rif* gene product. If both *stl* and *rif0* mutations affect the same cistron, the episome would fail to supply the *stl*$^+$ gene product that renders the cell sensitive to the drug. The merodiploids would therefore remain resistant to streptolydigin like their F$^-$ parents. That this is indeed the case is shown by the results presented in Table 4. It should be pointed out that some of the *rif0* mutations employed might exhibit the polar effect on the expression of the neighboring gene(s) that would complicate

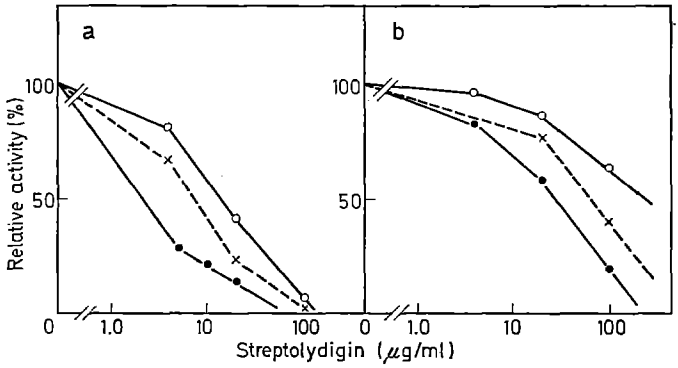

Fig. 1 a and b. Effect of *rif* (or *stv*) mutations on the sensitivity of RNA polymerase to streptolydigin. Cultures were grown in PG medium at 37° C to the late log phase, and crude extracts (0.5 unit) were used for each enzyme assay. Streptolydigin was added to the enzyme before addition of other components of the reaction mixture. a) *rif* (or *stv*) mutants obtained from strain KY 1342 or KY 1340 carrying the wild-type genes (*rif*$^+$ *stl*$^+$) for RNA polymerase. ×---×, KY 1342 (*rif*$^+$); ○—○, KY 1380 (*rif-291*); ●—●, KY 1375 (*stv*-362). b) *rif* mutants obtained from strain KY 1345 carrying the streptolydigin resistance gene (*stl-4*) for RNA polymerase. ×---×, KY 1345 (*rif*$^+$ *stl-4*); ○—○, KY 1376 (*rif-280 stl-4*); ●—●, KY 1378 (*rif-304 stl-4*)

Table 4. Cistron analysis between *stl* and *rif* mutations

Strain	*stl* or *rif* allele on		Stl phenotype[a]
	Chromosome	Episome (KLF10)	
KY1343	*stl-1*	—	Resistant
KY1343/KLF10	*stl-1*	*rif*+	Sensitive
	stl-1	*rif0-12*	Resistant
	stl-1	*rif0-14*	Resistant
	stl-1	*rif0-16*	Resistant
	stl-1	*rif0-17*	Resistant
	stl-1	*rif0-20*	Resistant
KY1348	*stl-7*	—	Resistant
KY1348/KLF10	*stl-7*	*rif*+	Sensitive
	stl-7	*rif0-20*	Resistant

[a] Stl phenotype was determined by streak tests on PG medium containing 100 μg/ml of streptolydigin at 37°C. Diploidy was confirmed by cross streaking cells of each F' strain against those of W3949 (F$^-$ *argH*) on minimal agar and the resulting F' cells were further checked for the *rif0* marker by the procedure described by Austin and Scaife (1970).

the interpretation of these results. However, it seems rather unlikely that all the *rif0* mutations tested are polar, particularly because they carry an amber suppressor gene *(supE)* that would suppress many of the polar mutations.

Fine Mapping of stl and rif Mutations

To examine the relative location as well as the possible clustering of *stl* and *rif* mutations on the chromosome, transductional mapping with phage P1 was performed employing a number of streptolydigin and rifampicin resistant mutants.

Table 5. Transductional mapping of *stl-1* and *rif* mutations

a) Donor (*rif* mutants of 2016): + + *rif* *purD*
Recipient (KY1344): *argE* *stl-1* + +

Selection	Unselected marker			No. of transductants obtained with donor carrying the *rif* alleles					
	stl	*rif*	*purD*	#266	#267	#268	#269	#270	#271
argE+	1[a]	1	1	23	27	28	24	30	34
	1	1	0	78	87	94	72	90	87
	1	0	1	0	1	1	1	2	0
	1	0	0	2	3	0	5	7	5
	0	1	1	0	0	0	0	0	1
	0	1	0	0	0	0	0	0	0
	0	0	1	10	3	6	1	1	2
	0	0	0	145	133	123	154	122	124
			Total	258	254	252	257	252	253

b) Donor (KY7302): + + *rif-252* *thiA*
Recipient (KY1344): *argE* *stl-1* + +

Selection	Unselected marker			No. of transductants obtained
	stl	*rif*	*thiA*	
argE+	1[a]	1	1	31
	1	1	0	20
	1	0	0	3
	0	1	0	1
	0	0	1	1
	0	0	0	67
			Total	123

All *rif* mutants used were obtained spontaneously and produced rifampicin-resistant RNA polymerase. Transduction and tests for unselected markers were performed as described under Materials and Methods.

[a] 1 represents markers from the donor and 0 represents those from the recipient. (This applies also for all subsequent tables.)

First, several rifampicin-resistant mutants were isolated independently from strains carrying *purD* or *thiA* and four point mapping with respect to *argE*, *rif*, *stl* and *purD* (or *thiA*) was carried out with the streptolydigin-resistant mutant carrying *argE* (KY 1344 *stl-1*) as the recipient. The results summarized in Table 5 indicate that all the *rif* mutations tested are clustered within a very short segment of the chromosome, and are also closely linked to the *stl-1* mutation, the probable gene order being *argE-stl-1-rif-thiA-purD*. The frequency of separation of these *rif* mutations (from *stl-1*) was no more than 4%. Similar results were obtained when double mutants carrying both *stl-1* and *rif* were used as the donor (Table 6).

In the second series of experiments, reciprocal transduction was carried out with another double mutant carrying *stl* and *rif* mutations. As seen in Table 7,

Table 6. Transductional mapping of *stl-1* and *rif* mutations using double mutants (*stl-1, rif*) as the donor

Donor (*rif* mutants of KY1344): *argE* *stl-1* *rif* +
Recipient (KY1368): + + + *purD*

Selection	Unselected marker			No. of transductants obtained with donor carrying the *rif* alleles			
	rif	*stl*	*argE*	#272	#273	#274	#275
purD+	1	1	1	32	544	60	90
	1	1	0	142		389	365
	1	0	1	0	20	1	1
	1	0	0	5		6	13
	0	1	1	0	1	0	0
	0	1	0	1		0	0
	0	0	1	0	498	1	5
	0	0	0	106		262	238
			Total	286	1063	719	712

All *rif* mutants used were obtained spontaneously from strain KY1344 and were found to produce rifampicin resistant RNA polymerase.

Table 7. Reciprocal transduction mapping of *stl-4* and *rif-276*

a) Donor (KY1342): + + + *thiA*
Recipient (KY1347): *argE* *rif-276* *stl-4* +

Selection	Unselected marker			No. of transductants obtained
	rif	*stl*	*thiA*	
argE+	1	1	1	38
	1	1	0	29
	0	0	0	53
			Total	120

b) Donor (KY1352): *rif-276* *stl-4* +
Recipient (KY1368): + + *purD*

Selection	Unselected marker		No of transductants obtained
	stl	*rif*	
purD+	1	1	483
	1	0	4
	0	1	0
	0	0	350
		Total	837

The *rif-276* mutation was introduced into strain KY1345 (stl-4) after treatment with ethylmethane sulfonate.

Table 8. Reciprocal transduction mapping involving *stl-1* and *stl-4*

a) Donor (KY1352): *rif-276* *stl-4* +
Recipient (KY1369): + *stl-1* *purD*

Selection	Unselected marker		No. of transductants obtained
	stl	*rif*	
purD$^+$	1 or 0[a]	1	147
	1 or 0	0	121
		Total	268

b) Donor (KY1369): + + *stl-1* *purD*
Recipient (KY1347): *argE* *rif-276* *stl-4* +

Selection	Unselected marker			No. of transductants obtained
	rif	*stl*	*purD*	
argE$^+$	1	1 or 0[a]	1	1
	1	1 or 0	0	173
	0	1 or 0	1	23
	0	1 or 0	0	67
			Total	264

[a] All transductants tested were resistant to streptolydigin and were not examined whether they carry the donor or recipient *stl* allele.

the two mutations *stl-4* and *rif-276* are extremely closely linked and crossovers between the two markers were observed only in 4 among 957 transductants tested. It should also be noted that *rif-276* is probably located between *stl-4* and *argE*.

Since the *rif-276* mutation appears to be located toward *argE* from the *stl* marker used in contrast to other *rif* mutations, it became important to find out the relative location of *stl-1* and *stl-4*. Another series of reciprocal transduction experiments were thus carried out. As shown in Table 8, no crossover between *stl-1* and *stl-4* that would give rise to streptolydigin-sensitive recombinant was observed among 532 transductants tested (*argE*$^+$ and *purD*$^+$ included). Thus, the two *stl* mutations must be located very close to each other with a frequency of separation of 0.4% or less. Fig. 2 shows the relative locations of all the *stl* and *rif* mutations described above.

Identification of the Altered Subunit of the stl-1 Mutant RNA Polymerase

In order to identify the altered subunit of the *stl-1* mutant RNA polymerase, attempts were made to reconstitute the active enzymes from mixed subunits of streptolydigin-sensitive and resistant polymerases. It was first shown that RNA polymerase and its subunits can be purified from the *stl-1* mutant (KY 1344) by the same procedures used for the wild-type enzyme, and the reconstituted enzyme from isolated subunits exhibits quantitatively similar resistance to streptolydigin

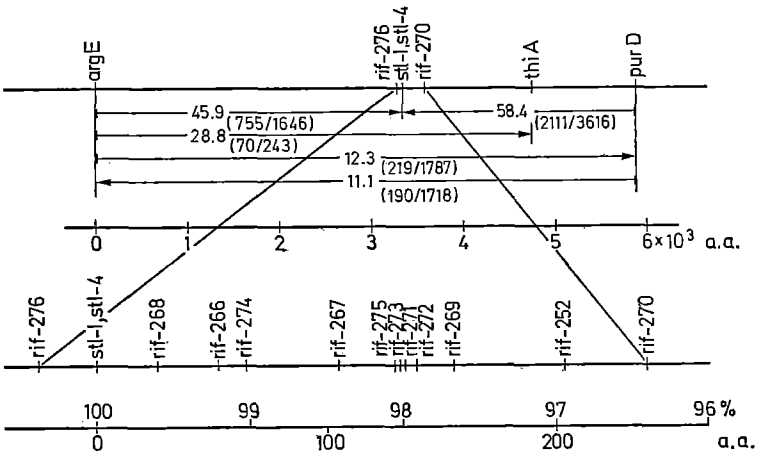

Fig. 2. Relative locations of the *stl* and *rif* mutations mapped by P1 phage transduction. Distances were calculated from the data presented in Tables 5–8. Values indicate co-transduction frequencies (%) between the markers involved as calculated from the numbers of transductants examined that are given in parentheses. Number of amino-acid residues that can be coded was estimated, assuming 0.015% recombination per amino-acid residue (Yanofsky *et al.*, 1967)

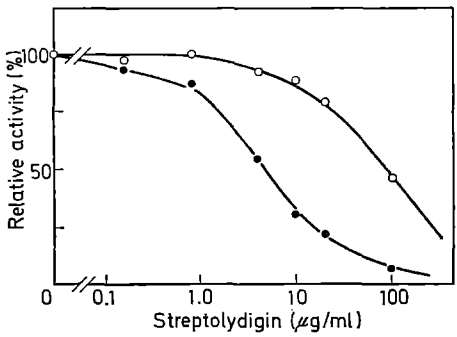

Fig. 3. Streptolydigin resistance of RNA polymerase from the *stl-1* mutant (KY 1344). Cultures were grown in PG medium at 37° C to the late log phase and RNA polymerase was purified from frozen cells as described in Materials and Methods. Three units of the enzyme was used for each assay with T7 DNA (7 μg) as template. Streptolydigin was added to the enzyme before addition of other components of the reaction mixture. Reaction was run for 15 min at 37° C. ●—●, KY1330(*stl*⁺); ○—○, KY1344(*stl-1*)

to that observed with crude extract or purified native enzyme (Fig. 3). Subunits were then separated from the mutant and wild-type RNA polymerase.

In the first experiment, chromatography of the urea-treated polymerase on a DEAE-Sephadex A-50 column separated the β' subunit from the mixture of α and β subunits. Fig. 4 shows the patterns of the two fractions thus obtained as analyzed by SDS-polyacrylamide gel electrophoresis. For reconstitution of the active enzyme, the two fractions were mixed at the stoichiometric ratio of each

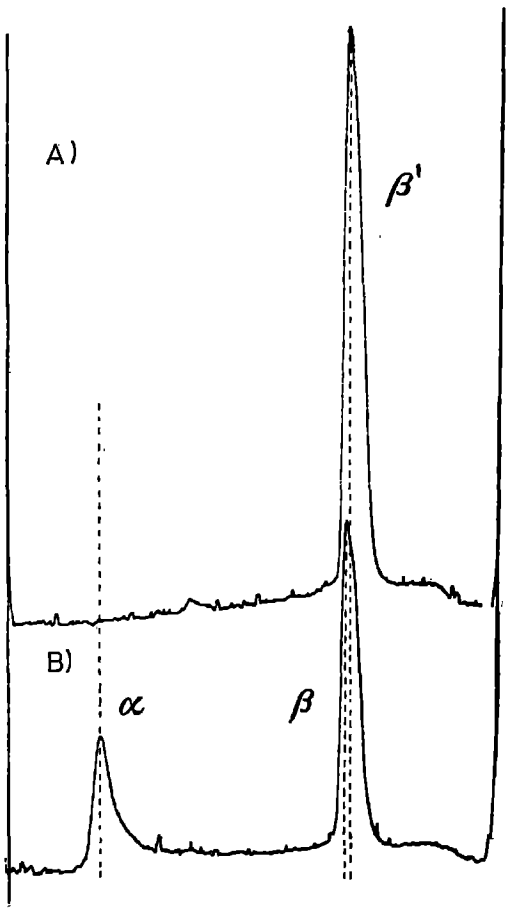

Fig. 4A and B

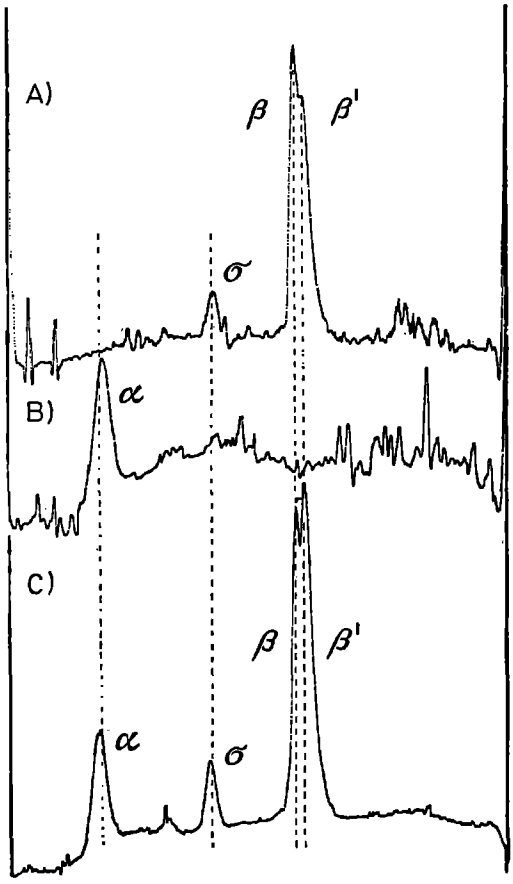

Fig. 5A—C

Fig. 4A and B. SDS-polyacrylamide gel electrophoresis patterns of the subunit fractions obtained by DEAE sephadex A-50 column chromatography. Purified RNA polymerase from strain KY 1330 (3 mg protein) was treated with 6 M urea at 30° C for 30 min in the standard dissociation buffer (Ishihama, 1972), applied onto DEAE sephadex A-50 column (1×30 cm), and was eluted with a linear gradient from 0.05 to 0.6 M KCl in the same buffer without $MgCl_2$. Aliquots (0.1 ml) of appropriately combined fractions were treated with 1% SDS at 30° C for 30 min, and were applied onto polyacrylamide gel containing 0.1% SDS according to the procedure of Shapiro *et al.* (1967). Gels were stained with Coomassie Brilliant Blue and were scanned by Joyce-Loeble microdensitometer MK-III. A) β' subunit fraction. B) $(\alpha+\beta)$ subunit fraction

Fig. 5A—C. SDS gel electrophoresis patterns of the subunit fractions obtained by Sephadex G-200 column chromatography. Wild-type RNA polymerase treated with urea as in Fig. 4 was filtered through Sephadex G-200 column (1×50 cm) in the standard buffer. Aliquots of each fraction were analyzed by SDS-gel electrophoresis as in Fig. 4. A) $(\beta+\beta')$ subunit fraction. B) α subunit fraction. C) Holo enzyme

subunit as found in the native enzyme ($\alpha:\beta:\beta' = 2:1:1$), and the mixture was dialyzed against the standard reconstitution buffer to gradually remove urea. The σ subunit was not added to the mixture, since it has no effect on the response of the polymerase to the drug. As can be seen in Table 9a, when the α and β subunits come from the resistant mutant, the reconstituted enzymes were always as resistant to streptolydigin as the native mutant enzyme irrespective of the source of the β' subunit. Thus, α and/or β but not β' subunit appeared to be altered in the *stl-1* mutant.

In the second experiment, gel filtration of the urea-treated polymerase through Sephadex G-200 successfully separated α from a mixture of β and β' subunits (Fig. 5). As shown in Table 9b, when the β and β' subunits from the mutant are used, the reconstituted enzymes are just as resistant to the drug as the native mutant enzyme whatever the source of the α subunit.

Thus, the results of these two reconstitution experiments clearly indicate that the β subunit of RNA polymerase is altered by the *stl-1* mutation. This conclusion is in agreement with the result of reconstitution experiment obtained by Heil and Zillig (1970) with another streptolydigin-resistant mutant.

Table 9. Reconstitution of RNA polymerase from the mutant (*stl-1*) and wild-type subunits

a) ($\alpha+\beta$) and β' subunit fractions

Source of subunit fraction		Residual enzyme activity (%) with streptolydigin	
($\alpha+\beta$)	β'	10 μg/ml	50 μg/ml
wild	wild	47.6	21.5
wild	mutant	44.9	23.5
mutant	wild	84.4	59.3
mutant	mutant	89.0	67.5

b) α and ($\beta+\beta'$) subunit fractions

Source of subunit fraction		Residual enzyme activity (%) with streptolydigin	
α	($\alpha+\beta'$)	10 μg/ml	50 μg/ml
wild	wild	52.1	21.8
wild	mutant	94.1	78.1
mutant	wild	47.6	19.3
mutant	mutant	92.1	75.6

Subunit fractions were obtained from the *stl-1* mutant (KY1344) and wild-type (KY1330) as described in Materials and Methods and legends to Figs. 4 and 5. Activity of reconstituted enzymes was assayed using deoxyAT copolymer (0.7 μg) as template, and incubation carried out for 30 min at 37°C. Amount of protein used was about 10 μg for each subunit combination. The activities are represented as the relative values compared to those in the absence of streptolydigin and the 100% activities for each combination ranged from 1600 to 22000 counts/min and from 20 to 60% of the activities of the untreated enzyme.

Discussion

Streptolydigin, an antibiotic produced by *Streptomyces lydigus*, inhibit RNA synthesis by interacting with the DNA-dependent RNA polymerase (Siddhikol *et al.*, 1969; Schleif, 1969). Addition of the antibiotic even after RNA synthesis has started results in an immediate cessation of further polymerization of nascent RNA chains, and the inhibition has been shown to be exerted primarily on the formation of the phosphodiester bond (Cassani *et al.*, 1971; von der Helm and Krakow, 1972). On the other hand, rifampicin (and presumably also streptovaricins) inhibits the initiation of RNA synthesis by blocking the formation of the preinitiation complex (Sippel and Hartmann, 1970; Bautz and Bautz, 1970). Similar modes of action for rifampicin and streptovaricins have also been suggested by the frequent occurrence of mutants resistant to both antibiotics (Yura and Igarashi, 1969). Thus, streptolydigin and rifampicin (or streptovaricins) inhibit the distinct but closely related reactions in the sequential events of RNA synthesis.

All the streptolydigin-resistant mutants examined carrying an F14 or KLF10 episome were found to be sensitive to streptolydigin due to the production of the drug-sensitive as well as resistant RNA polymerase. This fact, taken together with the results of enzyme reconstitution experiments, establishes that the gene determining the structure of β subunit of RNA polymerase is located on either of these episomes. It is also apparent that these *stl* mutant alleles are recessive to the wild-type *stl*$^+$ allele. Although our previous studies on streptovaricin resistance revealed the occurrence of dominant as well as recessive *stv* mutations (Yura and Igarashi, 1968), this does not imply any basic difference in the mechanism of dominance or recessiveness between *stl* and *stv* (or *rif*) mutations. Some of the merodiploids carrying mutant and wild-type alleles (*stv*/*stv*$^+$) may or may not grow in the presence of streptovaricins depending on the concentration used. In other words, the dominance is partial or incomplete. Such a situation has indeed been found with some of the rifampicin-resistant mutants (Knüsel and Schiess, 1970).

The rifampicin binding site on the polymerase is presumably located on the β subunit, since one of the rifampicin-resistant mutants was reported to produce polymerase whose β subunit shows an altered mobility in electrophoresis (Rabussay and Zillig, 1969). The structural alteration of the β subunit was further substantiated by the enzyme reconstitution experiments with a rifampicin resistant mutant and also with a streptolydigin-resistant mutant (Heil and Zillig, 1970). The direct demonstration of the altered β subunit in RNA polymerase from the *stl-1* mutant as well as the results of cistron analysis and of fine genetic mapping involving a number of *stl* and *rif* mutations point to the conclusion that all the *stl* and *rif* mutations examined affect one and the same cistron determining the structure of the β subunit of RNA polymerase. In view of the specific modes of action of the two antibiotics employed, this conclusion leads us to propose that the β subunit is directly responsible for catalyzing both the initiation and the elongation steps of RNA synthesis.

Extensive data on transductional mapping of the *stl* and *rif* mutations revealed that all the *rif* mutations tested are located rather close to *stl-1* (and also to *stl-4*). Assuming that the recombination frequency per amino acid residue

is 0.015% (Yanofsky *et al.*, 1967), the distance between *stl-1* and *rif* may be in the range of 30 to 300 amino acid residues on the β polypeptide, depending on the particular *rif* allele used. Since the β polypeptide contains about 1400 amino acid residues, these results suggest that all the *stl* and *rif* mutations are localized within a short segment of the structural gene for the β subunit. The close linkage between *stl* and *rif* as well as the clustering of independently occurring *rif* mutations was also reported recently by Mindlin *et al.* (1972). In contrast, other workers mapped some *rif* mutations at some distance from *stl* or other *rif* mutations, or even at the different cistron (Sokolova *et al.*, 1970; Marshall and Gillespie, 1972). Although the present results are not inconsistent with the latter findings, they seem to indicate that *rif* mutations usually occur within a narrow region on the β cistron ranging perhaps hundreds of nucleotides apart. This region might code for amino acid residues that are folded together to form the rifampicin binding site. As far as *stl* mutations are concerned, two independent mutations examined were extremely closely linked, but further mapping of additional mutants are required to see whether sites of *stl* mutations also constitute a cluster on the β cistron.

It should be noted that the majority of *rif* mutations was mapped between *stl-1* and *purD*, whereas one mutation *(rif-276)* appeared to be located between *argE* and *stl-1*, namely at the opposite side of *stl-1* (see Fig. 2). Thus, the mutation sites affecting sensitivities of the polymerase to rifampicin and streptolydigin appear to overlap at least partially to each other. If we assume that the mutational sites on the β cistron correspond to the drug-binding sites on the β polypeptide of the polymerase, the present data suggest that the rifampicin and streptolydigin binding sites are close to each other presumably with partial overlap. Simultaneous alterations of the polymerase in sensitivity to streptolydigin and to rifampicin (or streptovaricins) as a result of single mutation as revealed by the present study might be explained on the basis of this overlapping. However, as will be described elsewhere, binding of rifampicin to the polymerase could not be competed by excess amounts of streptolydigin (Iwakura *et al.*, in preparation).

The initiation of RNA synthesis and the subsequent elongation of nascent RNA chains have been believed to be catalyzed by the enzyme sites which are so constructed as to meet closely to each other. Since rifampicin and streptolydigin inhibit the initiation and the elongation steps, respectively, the closeness between the two drug-binding sites on the enzyme as suggested by the close linkage of the mutational sites is also in line with this general notion on the structure-function relationship of this enzyme. At any event, further studies will be required to find out the precise relationship between the drug-binding sites and the active centers on the enzyme on one hand, and that between those enzyme sites and sites on the β cistron affected by *rif* or *stl* mutations on the other.

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