JOURNAL OF BACTERIOLOGY, Oct., 1966
Vol. 92, No. 4

Printed in U.S.A.

Biochemical and Genetic Characterization of a Mutant of *Escherichia coli* with a Temperature-Sensitive Valyl Ribonucleic Acid Synthetase[1]

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Received for publication 19 April 1966

BÖCK, AUGUST (Purdue University, Lafayette, Ind.), LIA EIDLIC FAIMAN, AND FREDERICK C. NEIDHARDT. Biochemical and genetic characterization of a mutant of *Escherichia coli* with a temperature-sensitive valyl ribonucleic acid synthetase. J. Bacteriol. **92**:1076–1082. 1966.—To test our conclusion that *Escherichia coli* mutant I-9 possesses a valyl soluble ribonucleic acid (sRNA) synthetase that functions in vivo at 30 C but not at 37 C, measurements were made by use of the periodate method, of the level of charged valyl sRNA in this strain. A shift of temperature from 30 to 40 C resulted in a rapid discharging of valyl sRNA coordinate with the cessation of protein synthesis; at the same time, other species of sRNA, such as those for leucine, became fully charged. Identical results were obtained with a derivative of I-9 with relaxed ribonucleic acid (RNA) control. When P1 phage were grown on wild cells and then used at low multiplicities of infection to transduce temperature-resistant growth into I-9, complete cotransduction of normal valyl sRNA synthetase occurred. By means of the interrupted-mating technique, the structural gene for valyl sRNA synthetase was located on the *E. coli* chromosome map and found to be near *thr*, one-fifth of the length of the chromosome removed from the structural genes for the isoleucine-valine biosynthetic enzymes. Therefore, (i) the major valyl sRNA synthetase activity of I-9 appears to be temperature-sensitive in vivo, (ii) relaxed amino acid control over RNA synthesis does not appear to be a consequence of a normal charging of sRNA with a substitute molecule, and (iii) one structural gene for valyl sRNA synthetase is located on the *E. coli* chromosome not closely linked to the cistrons for the valine-biosynthetic enzymes.

The isolation of conditionally expressed mutants of *Escherichia coli* with altered aminoacyl soluble ribonucleic acid (sRNA) synthetases (2, 4) made it possible to correlate in vitro studies of these enzymes with their participation in protein synthesis and regulatory processes in vivo.

Incubation of I-9, a strain with an altered valyl sRNA synthetase activity (2), at a temperature which allows a restricted rate of protein synthesis results in the derepression of valine-repressible enzymes (3). This suggests that, at least in the case of the valine-biosynthetic enzymes, the amino acid must be activated or perhaps even attached to its cognate sRNA to be able to act as corepressor. Also, ribonucleic acid (RNA) synthesis in I-9 does not continue when protein synthesis is completely inhibited by shifting the cells from 30 to 40 C, even when chloramphenicol (40 μg/ml) is added to the cultures. This result indicates that amino acids must be activated in a stringent strain to permit RNA synthesis.

Clearly, these conclusions are valid only if I-9 possesses a valyl sRNA synthetase that operates in vivo at 30 C but becomes rapidly inactivated at temperatures above 36 C. Confirmation of this fact by in vitro studies of the enzyme is not possible, for extracts of I-9 fail to show activity even when prepared from cells never exposed to the restrictive temperature (2). On the other hand, preliminary genetic analysis suggested a common genetic basis for temperature sensitivity and the changed enzyme. We have assumed that, as has been found for several temperature-sensitive mu-

[1] Presented in part at the 66th Annual Meeting of the American Society for Microbology, Los Angeles, Calif., 1–4 May 1966.

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tants (e.g., 2, 15), the altered enzyme is so labile that its in vitro activity at any temperature is not an accurate measure of its in vivo activity at that temperature. Therefore, the present study was undertaken to measure directly the concentration of the product of valyl sRNA synthetase action in I-9 at permissive and restrictive temperatures. Another goal of the work was to confirm the genetic identity of the temperature sensitivity and the altered synthetase activity of I-9, and, further, to establish the chromosomal location of this gene.

MATERIALS AND METHODS

Organisms. The prototrophic strain of *E. coli*, KB, and the isolation of its temperature-sensitive derivative, I-9, have been described previously (2). Several auxotrophic derivatives of I-9 were obtained by the method of Gorini and Kaufman (6): I-9 *ilva⁻ leu⁻ met⁻ str-r;* I-9 *ilva⁻ leu⁻ arg⁻ str-r;* I-9 *ilva⁻ leu⁻ str-r;* and I-9 *his⁻ ser⁻*. In addition, strain Re24 was isolated as a recombinant from a mating between an I-9 derivative and a K-10 derivative; it possesses the temperature sensitivity (and altered valyl sRNA synthetase) of I-9 and the RC^{rel} character of K-10. The following abbreviations of genetic markers are used: *ilva*, isoleucine-valine; *leu*, leucine; *met*, methionine (probably the *A* cistron); *str*, streptomycin; *his*, histidine; *ser*, serine-glycine; *RC*, RNA control; *ts*, the temperature-sensitivity gene of I-9 (ts^+, ability to grow at 37 C; ts^-, inability to grow at 37 C); *s*, sensitive; *r*, resistant; *rel*, relaxed control; *st*, stringent control; +, ability and, −, inability, to synthesize the indicated compound.

Media. The minimal medium used consisted of salt solution P (5) supplemented with 0.6% $(NH_4)_2SO_4$ and 0.4% glucose; the rich medium (TGYE) contained 1% tryptone, 0.5% yeast extract, and 0.2% glucose. For the mating experiments, TGYE was supplemented with 1% NaCl (TGYES).

Crude aminoacyl sRNA synthetases. The synthetase preparations were cell-free extracts from which nucleic acids had been removed by the method of Kelmers et al. (7).

Preparation and oxidation of sRNA. A modification of the procedure of Morris and DeMoss (11) was used to isolate sRNA and to determine its degree of protection against periodate oxidation. Cells were grown to an optical density (OD), at 420 mμ, of 2.0, chilled rapidly, and centrifuged; the pellet was suspended in 2 ml of 0.05 M sodium acetate (*p*H 5.5), 0.06 M KCl, and 0.01 M $MgCl_2$. The cells were broken by treatment with a Branson sonic oscillator for 1 min at setting 3. Phenol extraction and ethyl alcohol precipitation were carried out as described by Morris and DeMoss (11).

The precipitate was collected by centrifugation and dissolved in 2 ml of 0.01 M sodium acetate (*p*H 5.2); this solution was separated into two 1-ml samples. To one sample, 0.1 ml of 10^{-1} M $NaIO_4$ was added, and the mixture was incubated for 50 min at 25 C in the dark. Excess periodate was then destroyed by adding a twofold concentration of glucose and continuing the incubation for 5 min. Treated and untreated samples were dialyzed overnight against distilled water at 4 C. They were then stripped of attached amino acid either by the enzymatic stripping method of Muench and Berg (12) or by incubation in 0.1 M tris(hydroxymethyl)aminomethane (Tris) chloride buffer (*p*H 8.8) at 37 C for 2 hr. The enzymatic stripping method is more effective, particularly for the unusually stable ester bond of valyl sRNA.

The RNA content of the samples was determined by measuring their OD at 260 mμ, assuming 24 OD units to be equal to 1 mg of RNA per ml. The specific acceptor activity of 150- to 200-μg portions of the oxidized and enzymatically stripped untreated samples was determined by charging the sRNA to completion by the method of Muench and Berg (12), except for the use of 50 μmoles Tris chloride (*p*H 7.3). For nonenzymatically stripped sRNA, the reaction mixture was: Tris chloride (*p*H 7.3), 50 μmoles; $MgCl_2$, 5 μmoles; KCl, 5 μmoles; glutathione (*p*H 6.8), 1 μmole; dipotassium adenosine triphosphate (ATP; *p*H 6.8), 1 μmole; L-amino acid-C^{14} (20 μc/μmole), 0.01 μmole; and crude extract protein, 10 μg (final volume, 0.5 ml). Samples without ATP were used as controls. To assure that the reaction had gone to completion, incubation (37 C) was carried out in duplicate, one sample being incubated for 15 min, the other for 20 min. The reaction was stopped by chilling and adding 2.5 ml of 10% trichloroacetic acid containing 2 mg/ml of DL-amino acid. The samples were filtered through membrane filters (Schleicher and Schuell, B4); they were washed twice with 5-ml portions of 5% trichloroacetic acid containing 2 mg/ml of amino acid and then with 5 ml of 67% ethyl alcohol (−20 C). The filters were glued onto planchets, and their radioactivity was determined in a Nuclear Chicago thin end-window gas flow counter.

The percentage of sRNA which existed in the charged form in vivo is given by the formula: (counts per minute per microgram of oxidized RNA)/(counts per minute per microgram of untreated RNA) × 100.

Radioactivity determinations. Incorporation of C^{14}-uracil and C^{14}-isoleucine into cell material was measured as described previously (2).

Transduction. Transduction experiments were performed by the procedure of Lennox (10). For the interrupted matings, the method of Taylor and Thoman (14) was followed. The matings were done at 30 C, since I-9 [converted into an F^- phenocopy (9)] served as the recipient. Non- and exconjugant Hfr cells were killed by including 200 μg/ml of streptomycin in the selective media.

RESULTS

Correlation of valyl sRNA charging with protein synthesis. The steady-state level of charged valyl sRNA in the wild strain (KB) grown at 30 C and at 40 C is shown in Table 1. The lower level at 40 C indicates that the temperature coefficient for valine attachment may be different from that for overall protein synthesis. The value for the temperature-sensitive mutant growing at 30 C was essentially the same as that for KB. Table 1 also

TABLE 1. *Effect of temperature and amino acid starvation on valyl sRNA charging*

Stain	Medium	Temp	Specific acceptance activity[a]	Valyl sRNA charging
		C		%
KB	Minimal + aa[b]	30	2.06 (1.74)	85
KB	Minimal + aa	40	2.34 (1.77)	76
I-9 *ilva*	Minimal + aa	30	2.90 (2.48)	86
I-9 *ilva*	Minimal + aa − valine	30[c]	4.22 (0.30)	7
I-9 *ilva*	Minimal + aa	40[d]	3.17 (0.73)	23

[a] Valine-accepting capacity of RNA isolated from each culture, expressed as counts per minute per microgram of ribonucleic acid. The number in parenthesis is the accepting capacity after periodate oxidation of the sample. All samples were stripped enzymatically before final acceptance assay.
[b] Mixture of 20 L-amino acids, 50 μg/ml of each.
[c] Incubation for 30 min in valine-deficient medium.
[d] Incubation at 40 C for 30 min.

shows the marked reduction in valyl sRNA charging when an isoleucine-valine requiring derivative of I-9 was incubated for 30 min in a medium lacking valine. Finally, exposure of the mutant to 40 C in a complete medium for 30 min resulted in nearly as great a reduction of valyl sRNA charging as did starvation for this amino acid.

Experiments were next done with strain I-9 to compare the time course of the decrease in valyl sRNA charging with the loss of ability to make protein at the restrictive temperature (39 to 40 C). Cultures of I-9 growing in minimal or in rich medium at 30 C were shifted to 40 C, and samples were removed at intervals after the shift, to be assayed for percentage of charged valyl sRNA. The decrease in charging was most rapid during the first 10 to 20 min after raising the temperature, even in rich medium amply supplied with valine (Fig. 1). The charging of leucyl sRNA was measured as a control; its degree of charging, after an initial slight drop, rose to near 100%, an expected consequence of the inhibition of protein synthesis.

Other cultures received supplements of C^{14}-labeled L-isoleucine before, during, or after the temperature shift to estimate the rate of protein synthesis. The ability of strain I-9 to make protein fell to near zero by 15 min after the temperature shift, in apparent correlation with the decrease in valyl sRNA charging (Fig. 2).

Effect of valyl sRNA charging on control of RNA synthesis. When strain I-9 is starved of an amino acid, RNA synthesis halts; in other words it is an *RC* stringent strain. We have previously shown that strain I-9 fails to overproduce RNA when protein synthesis is inhibited by exposure of the cells to 40 C, indicating that the mere presence of valine is insufficient for RNA synthesis if it cannot be activated (2). The data in Fig. 1, therefore, are consistent with the hypothesis proposed by Stent and Brenner (13) and by Kurland and Maaløe (8) that uncharged sRNA inhibits RNA synthesis. It has been suggested that strains with relaxed amino acid control over RNA synthesis overcome this inhibition by charging their sRNA

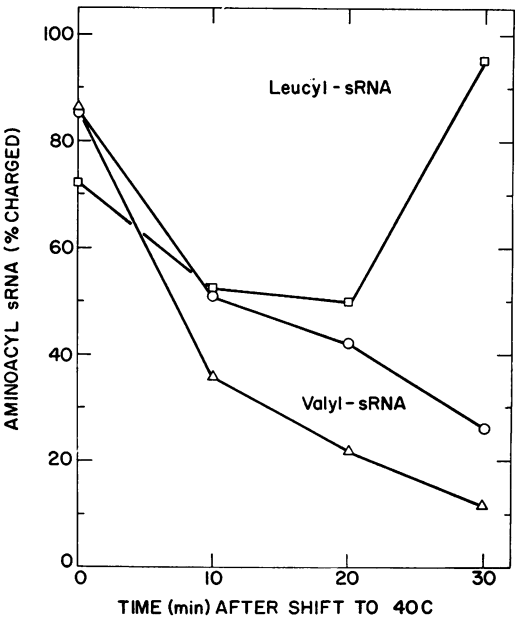

FIG. 1. *Effect of a shift to 40 C on the in vivo level of charging of sRNA in strain I-9. Samples were removed at 0, 10, 20, and 30 min from a culture of I-9 growing in rich medium, and the in vivo levels of valyl sRNA* (○) *and leucyl sRNA* (□) *were measured. Valyl sRNA* (△) *charging was also measured in a similar experiment employing minimal medium. Samples were stripped enzymatically before final acceptance assay. The total valine acceptance activity for the culture in rich medium was (in counts per minute per microgram of RNA): 0 min, 1.87; 10 min, 2.81; 20 min, 2.92; 30 min, 3.18); the values were similar for the culture in minimal medium.*

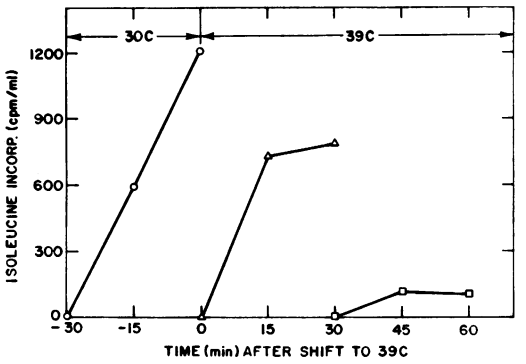

FIG. 2. *Effect of a shift to 39 C on the rate of protein synthesis in strain I-9. A culture of I-9 growing in minimal medium at 30 C was divided into three portions. To one* (○), *C^{14}-labeled L-isoleucine was added 30 min before the temperature shift, and samples were removed 15 and 30 min later to be assayed for radioactive protein. The second culture* (△) *received the same amount of C^{14}-labeled L-isoleucine at the time of the temperature shift, and samples taken 15 and 30 min later were taken for assay. The third culture* (□) *received the labeled amino acid 30 min after the temperature shift, and similar samples were taken for assay.*

with some as yet uncharacterized "catholic inducer" (1). The altered valyl sRNA synthetase of strain I-9 provides a useful tool for examining this hypothesis, since it permits one to block charging of one class of sRNA molecules under conditions where a supply of the amino acid by protein turnover cannot complicate the experiment.

Accordingly, a strain was constructed that possesses the altered valyl sRNA synthetase of I-9 together with the *RC* relaxed gene. Table 2 shows the strikingly different response given by strain I-9 (*ts*$^-$, *RC*st) and that by strain Re24 (*ts*$^-$,*RC*^{rel}) when the temperature was raised to 40 C: strain I-9 failed to accumulate nucleic acid; strain Re24 nearly doubled its content in 60 min. (Strain I-9 did exhibit some incorporation, indicating a small amount of turnover of nucleic acid at 40 C). The two strains differed in their response to the temperature shift just as in their response to amino acid starvation. Figure 3, however, shows that the temperature shift reduced valyl sRNA charging in strain Re24 exactly as in I-9.

It appears, then, that the relaxed cells produce RNA at 40 C despite their inability to attach valine, or any other metabolite, to the terminal adenosine residue of valine's sRNA.

Transduction of temperature sensitivity and valyl sRNA synthetase activity. The biochemical information presented in the preceding sections supports the belief that the temperature-sensitive growth response of strain I-9 is a consequence of its altered valyl sRNA synthetase activity. These two characteristics behaved as a single genetic unit in mating experiments reported earlier (2), but, to test this notion in another way, transduction experiments were performed with the generalized transducing phage P1kc. Phage were grown on the wild strain, KB, and then were used to infect auxotrophic derivatives of strain I-9 at very low multiplicities of infection (1.0 or less). Transductants were selected by plating on rich medium at 40 C, on minimal medium at 30 C, and on minimal medium at 40 C. Table 3 shows typical results from one such experiment. Temperature resistance was transduced about as readily as another chromosomal marker (*his*$^+$), and no double transductants (*ts*$^+$ *his*$^+$) were detected. Of the *ts*$^+$ transductants measured, all had gained normal wild-type levels of valyl sRNA synthetase, providing evidence for the genetic identity of these two characteristics.

Chromosomal location of valyl sRNA synthetase gene. To localize the structural gene for valyl sRNA synthetase on the chromosome map of *E. coli*, interrupted-mating experiments were performed at 30 C with F$^-$ phenocopies of different I-9 auxotrophs as recipients and K-10 *try*$^-$ as Hfr (Fig. 4). Prior to these experiments, the approximate sector of the map containing the *ts* marker had been roughly determined by linkage analysis in noninterrupted matings with the Hfr strains cited in Fig. 5. In the three interrupted matings, *ts*$^+$ entered the F$^-$ approximately 8 min after *leu*;

TABLE 2. *Incorporation of C^{14}-uracil by strains I-9 (ts$^-$ RCst) and Re24 (ts$^-$ RC^{rel}) after a shift to 40 C*

Time after temp shift	Uracil-2-C^{14} incorporated			
	Cells not prelabeled[a]		Prelabeled cells[b]	
	I-9	Re24	I-9	Re24
min				
0	—	—	1.00	1.00
10	1.44	7.65	0.97	1.25
20	1.69	14.60	0.90	1.41
30	2.24	20.30	0.91	1.63
60	3.44	30.60	0.88	1.96

[a] Radioactive uracil was added at zero-time to cultures in minimal medium containing 50 μg of uracil per ml. The data are expressed as millimicromoles of uracil incorporated by a 2-ml sample at an OD of 1.0.

[b] These cultures had been grown in minimal medium containing uracil-2-C^{14}. The data are normalized to the values at zero-time.

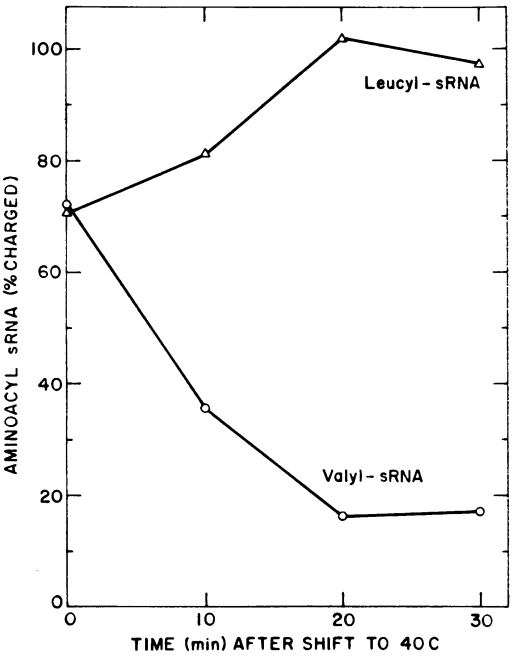

FIG. 3. *Effect of a shift to 40 C on the in vivo level of charging of sRNA in strain Re24. Samples were removed at 0, 10, 20, and 30 min from a culture of the relaxed, temperature-sensitive strain, Re24, growing in rich medium, and the in vivo levels of valyl sRNA* (○) *and leucyl sRNA* (△) *were measured. Samples were stripped by incubation in 0.1 M Tris buffer (pH 8.8) for 2 hr at 37 C. The total valine acceptance activity was (counts per minute per microgram of RNA): 0 min, 1.92; 10 min, 1.70; 20 min, 1.99; 30 min, 2.44.*

TABLE 3. *Cotransduction of temperature sensitivity and valyl sRNA synthetase activity*[a]

Selected marker	Transduction frequency per 10^7 recipients	No. of transductants with normal enzyme/no. tested
ts$^+$	116	35/35
his$^+$	280	—
ts$^+$ *his*$^+$	0	—

[a] Phage P1kc were grown on strain KB (*ts*$^+$ *his*$^+$ *ser*$^+$) and used to transduce a strain derived from I-9 (*ts*$^-$ *his*$^-$ *ser*$^-$). All 35 *ts*$^+$ transductants were *his*$^-$ *ser*$^-$. The multiplicity of infection was approximately 1.0.

the absolute time of entry was variable, probably as a result of differences in physiological state of the F$^-$ phenocopies. When corrected to 37 C, this value is equivalent to 2.5 min on the Taylor-Thoman (14) map. These results locate the structural gene (altered in I-9) for valyl sRNA synthetase at minute 87.

DISCUSSION

These results establish that, despite the failure to detect enzyme activity in vitro, valyl sRNA synthetase functions in vivo in I-9 cells growing at the permissive temperature, and fails to do so at the restrictive temperature. The preferential discharging of valyl sRNA is almost completed within 15 min at 40 C, and this time course is in excellent agreement with the time course of inhibition of protein synthesis and of derepression of valine repressible enzymes (3). Furthermore, cotransduction of wild-type valyl sRNA synthetase activity and temperature resistance provides evidence that a single mutation located in a structural gene for this enzyme renders the cells of I-9 temperture-sensitive.

Discharge of valyl sRNA was not complete at 40 C; 10 to 20% remained charged. The residual charging may have one of several explanations. (i) Very slight, but still detectable, incorporation of amino acids into protein continues at 40 C, and therefore one might postulate that a residual synthetase activity is responsible for the charging. (ii) If there is more than one species of sRNA with unique transfer functions for valine, discharge of any one of them would halt discharge of the others. (iii) If some minor species of valyl acceptor sRNA has no function in protein synthesis, it might not become discharged in the short time period of our experiments.

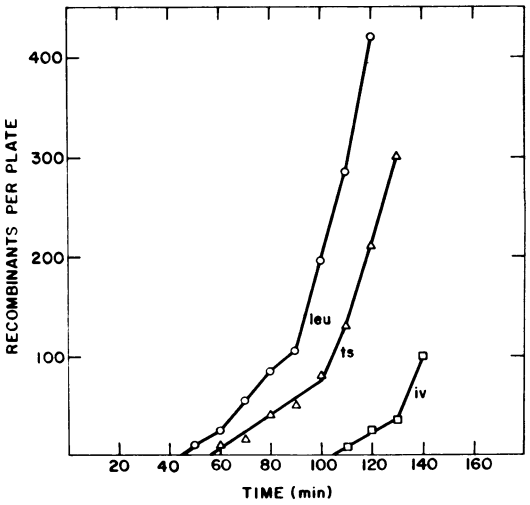

FIG. 4. *Time of entry of leu*$^+$*, ts*$^+$*, and ilva*$^+$ *markers in an interrupted mating at 30 C between strain I-9 (F*$^-$ *phenocopy; ilva*$^-$ *leu*$^-$ *str-r ts*$^-$*) and strain K-10 (Hfr; ilva*$^+$ *leu*$^+$ *str-s ts*$^+$*). Other interrupted matings, not shown, were performed with I-9 (F*$^-$ *phenocopy; ilva leu arg str-r ts*$^-$*) and with I-9 (F*$^-$ *phenocopy; ilva*$^-$ *leu*$^-$ *met*$^-$ *str-r ts*$^-$*).*

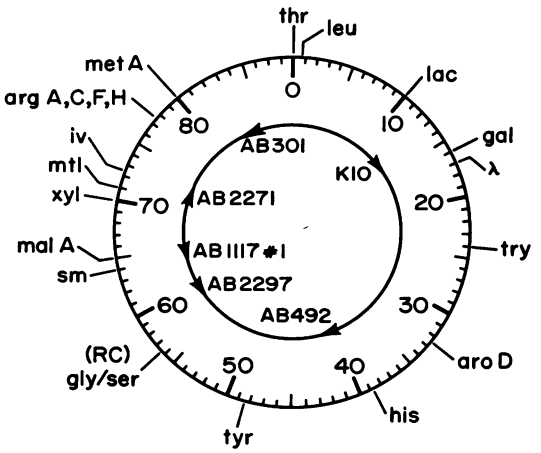

FIG. 5. *Genetic map of Escherichia coli K-12. This figure is redrawn from Taylor and Thoman (14). The outer circle shows the position of frequently used genetic markers, and is calibrated in minutes required for transmission to an F⁻ at 37 C. The inner circle indicates the points of origin and direction of transfer of Hfr strains. Only K-10 was used in this study.*

The derepression of valine-controlled enzymes in I-9 at restrictive temperatures has permitted the conclusion that valine connot regulate enzyme synthesis unless it interacts in some way with a functioning valyl sRNA synthetase. Recent (unpublished) measurements on extracts of I-9 cells revealed a considerable ability to catalyze valyl adenylate formation, suggesting that the rate-limiting process at high temperature is the sRNA attachment function of the synthetase. Therefore, the possibility that the valyl-enzyme complex or valyl adenylate-enzyme complex acts as a repressor is not very likely. Rather, it would seem that, to act as a corepressor, valine must be attached by means of valyl sRNA synthetase to one or more of its cognate sRNA molecules or to some still unknown aporepressor.

The results with strain Re24 show that cells with an RC^{rel} gene can overproduce RNA in spite of (and because of) an almost completely uncharged valyl sRNA. Therefore, it appears that (i) an active valyl sRNA synthetase activity is not required for relaxed production of RNA, (ii) the RC^{rel} locus does not code for some enzyme of loose specificity that can charge valyl sRNA with a "catholic inducer", and (iii) the RC^{rel} locus does not govern the production of a "catholic inducer," at least not one that acts by combining with sRNA. There does exist the possibility that *RC* affects the structure of RNA polymerase itself or the structure of regulating molecules other than sRNA. It now seems more fruitful, however, to consider biochemical mechanisms for RNA regulation that do not directly involve the charging level of sRNA.

The major initial significance of the mapping information presented in this study is the fact that the structural gene for valyl sRNA synthetase is not adjacent to the cluster of cistrons coding for the valine-biosynthetic enzymes. A similar map location has been reported by Yaniv et al. (15) for their independently isolated *ts* mutant with an altered valyl sRNA synthetase. A structural gene for at least one other aminoacyl sRNA synthetase has been mapped in our laboratory (*unpublished data*), and it is not adjacent to the valyl sRNA synthetase gene.

ACKNOWLEDGMENTS

We are greatly indebted to Barbara Maling for introducing us to the art of P1 transduction, and to Joan McCune for skillfully carrying out the transductions reported here.

This investigation was supported by grant GB-2094 from the National Science Foundation.

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