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Enzymes Producing 4-Thiouridine in *Escherichia coli* tRNA: Approximate Chromosomal Locations of the Genes and Enzyme Activities in a 4-Thiouridine-Deficient Mutant

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A previously described mutant of *Escherichia coli* which lacks 4-thiouridine in its tRNA was here shown to be deficient in factor A, one of the two proteins responsible for this thiolation of uridine. Addition of exogenous factor A restored the thiolating ability of extracts prepared from the mutant. The activities of the two thiolation proteins were governed by genes at two widely separated positions on the chromosome, as determined with F-prime merodiploids. The site governing factor A activity lay roughly in the region of the recently reported position of *nuv*, a gene controlling the production of 4-thiouridine in tRNA.

Recent reports by Ramabhadran et al. (13) and by Thomas and Favre (14) indicate that some mutants of *Escherichia coli* are resistant to irradiation in the near-UV range (3 to 400 nm) because they have tRNA which is deficient in 4-thiouridine (s^4U), a minor nucleotide in tRNA which absorbs in this same UV region. We show here that in one such mutant, s^4U is the only thionucleotide which is lacking and that the levels of the three other major thionucleotides are perfectly normal.

Although it was thought that the deficiency in s^4U lay in the specific thiolating system producing this tRNA modification, a direct demonstration has never been made. The transformation of uridine to s^4U in *E. coli* tRNA depends on the action of two separate proteins, factors A and C (1). This report indicates that, whereas factor C activities were identical in extracts prepared from mutant (s^4U^-) and parental cells, factor A activity was greatly depressed in the mutant strain. Production of s^4U was resumed in extracts from the mutant strain after addition of exogenous purified factor A prepared from wild-type *E. coli*.

Neither of the genes governing these enzymatic activities has ever been mapped. Using appropriate F-prime strains and either the gene dosage technique or correction of the lesion through introduction of F-prime material into the mutant by mating, gross localization of the two genes associated with factor A and factor C activities showed that they were widely separated on the *E. coli* chromosome. The marker for factor A activity was found to be in the same general region as the genetic marker *nuv*, related to s^4U deficiency and UV resistance, which was mapped in *E. coli* K-12 by Thomas and Favre (15).

MATERIALS AND METHODS

Bacterial strains. The strains used are listed in Tables 1 and 2. The F-prime merodiploids were obtained from the Coli Genetic Stock Center (CGSC), Yale University, New Haven, Conn., and were grown in M9 minimal medium (10) supplemented as indicated by CGSC. We are indebted to John Jagger for the mutant *E. coli* strain RJ-1 and its parent, *E. coli* B/r NC32; Philip E. Hartman supplied *E. coli* strain RW84; and a culture of *E. coli* KL96 was provided by Alan Peterkofsky.

Preparation of cell extracts for assay of thiolating activity. Cells were grown in nutrient broth at 30°C, harvested at an absorbance at 550 nm (A_{550}) of 1, ground with alumina, extracted with buffer A (50 mM tris(hydroxymethyl)aminomethane-chloride buffer [pH 7.8], 20% [vol/vol] glycerol, 5 mM $MgCl_2$, 5 mM 2-mercaptoethanol, and 1 mM ethylenediaminetetraacetate), and carried through dextran phase partition as previously described (1). The resulting $(NH_4)_2SO_4$ precipitate was dissolved, desalted on a Sephadex G25 column equilibrated with buffer B [50 mM tris(hydroxymethyl)aminomethane-chloride (pH 8.0), 5 mM $MgCl_2$, 50 mM KCl, and 5 mM 2-mercaptoethanol], and applied to a diethylaminoethyl-cellulose column equilibrated with buffer B. The KCl content of buffer B was raised to 80 mM for washing the column and to 0.25 M for elution of protein. This eluate, which now lacked the tRNA and about one-third of the inert protein, was assayed as described below.

Measurement of thiolating activity. In general, measurement of thiolating activity involves incubating the enzyme source with Mg^{2+}, ATP, [^{35}S]cysteine, 2-mercaptoethanol, and 100 A_{260} units of *E. coli* tRNA,

TABLE 1. *F-prime-carrying strains*

F-prime[a]	Point of origin	Strain designation	CGSC no.
13	Hfr13	W3747	5218
42	HfrP804	NH4104	4349
104	HfrH	KLF4/AB2463	4251
111	HfrJ4	KLF11/JC1553	4258
112	HfrRa-2	KLF12/JC1553	4260
116	HfrAB312	KLF16/KL110	4254
117	HfrH	KLF17/JC1553	4255
123	HfrBroda 7	KLF23/KL181	4256
126	HfrBroda 7	KLF26/KL181	4253
128	HfrP804	E5014	4288
129	HfrKL98	KLF29/JC1553	4280
133	HfrKL25	KLF33/JC1553	4265
140	HfrAB312	MAF1/JC1553	4289
141	HfrAB312	KLF41/JC1553	4248
142	HfrKL98	KLF42/KL253	4279
148	HfrPK191	KLF48/KL159	4302
150	KL96	DFF1/JC1553	4326
196	KL96	WH-1	4612
254	HfrOR11	ORF4/KL251	4282

[a] The extent of each F-prime factor may be found in reference 8.

TABLE 2. *Strains of E. coli*

Strain	Genotype	Source reference
W3747	*metB1 rel-1* deletion corresponding to F13	CGSC (4)
NH4104	*thr-1 leu-6 thi-1 his-4 proA2 uvrA6 lacY1 ara-14 supE44?*	CGSC
AB2463	*thi-1 argE3 his-4 leu-6 thr-1 proA2 recA13 mtl-1 xyl-5 ara-14 galK2 lacY1 str-31 tsx-33* λ^- *supE44*	CGSC
JC1553	*argG6 metB1 his-1 leu-6 mtl-2 xyl-7 malA1 gal-6 lacY1 str-104 tonA2 tsx-1* λ^r λ *supE44*	CGSC
KL110	JF1553 *thyA23*	CGSC
KL181	*thi-1, pyrD34 his-68 trp-45 recA1 mtl-2 xyl-7 malA1 galK35 str-118* λ^r λ	CGSC
E5014	*thi-1 rel-1? mal-24 spe-12 supE50* Δ*(proB-lac)5*	CGSC
KL253	KL191 *tyrA2*	CGSC
KL159	*thi-1 his-4 aroD5 proA2 recA1 xyl-5* λ^- *supE44?*	CGSC
WH-1	*his-45 trp-37 phoA4 recA1 str-144 val*^{r}*-116*	CGSC (5)
KL251	*thi-1 leu-6 metE70 tryE38 purE42 proC32 recA1 mtl-6 xyl-5 ara-14 lacZ36 str-109 azi-6 tonA23 tsx-67* λ^- *supE44*	CGSC
B/rNC32	*lac valS*(Ts) *rel*$^+$	J. Jagger (13)
RJ-1	B/rNC32, deficient in s^4U in tRNA	J. Jagger (13)
RJ-1-4	Isolate from RJ-1 culture, tRNA has A_{335} 0.13% that of A_{260}	This paper
KL96	Hfr *thi-1 rel-1* λ^-	A. Peterkofsky (7)
RW84	F$^-$ *edo-1 edd* Δ*(his-gnd) str*^{r}	P. Hartman (16)

which provides both an excess of potential sulfur-accepting sites and a known amount of unlabeled s^4U to act as carrier. After the incubation, the tRNA is reisolated and hydrolyzed, and the s^4U is purified by diethylaminoethyl-cellulose chromatography. Results are expressed as picomoles of ^{35}S incorporated per A_{335} unit of recovered s^4U in 30 min. Details are published elsewhere (1).

Assays of F-prime strains for variations in the s^4U-forming system. Cells were grown at 37°C in 500 ml of M9 medium suitably supplemented to maintain the F-prime factor and to satisfy auxotrophic requirements. They were harvested at an A_{550} of 1 and washed with cold glucose-free M9 solution. Each cell pellet was ground with an equal weight of alumina and extracted with buffer A containing 2 μg of ribonuclease-free deoxyribonuclease (Worthington Biochemicals Corp., Freehold, N. J.) per ml. Centrifugation at 105,000 $\times$ *g* yielded supernatant solutions that were dialyzed against 3 liters of cold buffer A for 2 h to remove inhibitors, and the protein concentrations were determined. Each extract was assayed as above in a short assay (10 min, to control nuclease activity), using 0.5- and 1-mg protein levels with and without supplements of purified factor A (15 U) or factor C (30 U). As a control, *E. coli* K-12 strain KL96 was grown and worked up in parallel with each group of three F-prime strains.

RESULTS

Thionucleotide pattern of tRNA's. The s^4U in *E. coli* tRNA has an A_{335} which is normally about 1.5 to 1.8% of the A_{260} (6). The UV spectrum of tRNA isolated from the mutant RJ-1 showed a level of s^4U far below normal. The A_{335} in 10 isolated colonies ranged from 0.13 to 0.4% of the A_{260}, as compared with 1.5% in the parent, *E. coli* strain B/r NC32. One of these isolates, strain RJ-1-4, consistently yielding a value of 0.13%, was used for these and the following studies.

Since cysteine is the sulfur donor for all of the different thionucleotides (11), a defect in cysteine metabolism might affect not only s^4U, but also the general thionucleotide pattern. To study this possibility, digests of tRNA prepared from cultures of RJ-1 and NC32 grown in [^{35}S]sulfate were chromatographed on cellulose phosphate columns (12). Aside from the obvious variation in the amount of s^4U in the two preparations, there was no marked difference between the thionucleoside patterns (Fig. 1). Therefore, it seemed probable that the mutation in strain RJ-1 specifically affected the activity of one or both of the proteins involved in s^4U formation.

Enzymatic activity in cell extracts. Crude cellular extracts are difficult to use for measurement of thiolation enzymes because they contain both inhibitors and nucleases. Alumina-ground cells were therefore first extracted and fraction-

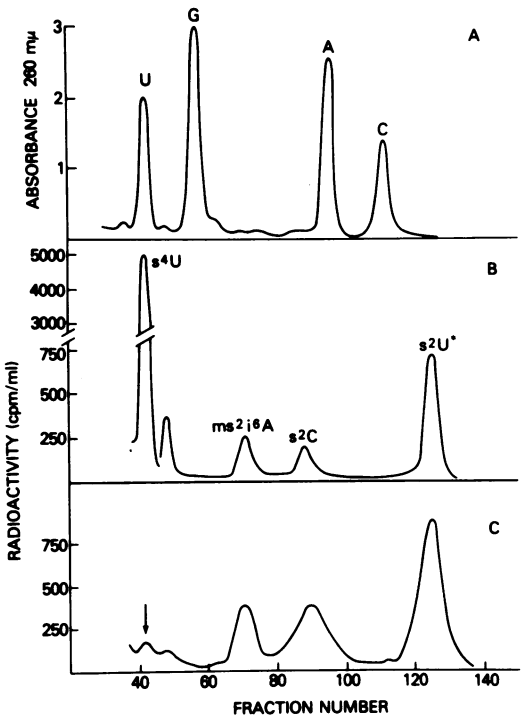

FIG. 1. *Distribution of thionucleosides in normal and mutant strains. Cells were grown to late log phase at 30°C in 1 liter of M9 medium (10) containing 1 mCi of carrier-free $H_2{}^{35}SO_4$. Packed cells were ground with alumina, extracted with 10 ml of buffer B, and centrifuged at 105,000 × g for 3 h. The supernatant fluid was deproteinized with 88% phenol, and the aqueous phase was precipitated with 2 volumes of ethanol. The tRNA pellet was freed of amino acids by incubation in 0.1 M glycine buffer (pH 10.5) for 1 h at 37°C and precipitated with ethanol. The precipitated material was dialyzed against water, hydrolyzed with 0.3 N KOH, dephosphorylated, and chromatographed on a cellulose phosphate column as previously described (13). Samples of 1 ml were counted in 10 ml of Bray solution (3) in a liquid scintillation counter. (A) UV absorbance profile for both columns (abbreviations: U, uridine; G, guanosine; A, adenosine; C, cytidine). (B) Thionucleoside pattern for parental strain, NC32; input, 52,500 cpm, 93% recovery. (C) Thionucleoside pattern for mutant strain RJ-1, input, 200,000 cpm, with 80% recovery. Thionucleoside peaks are, in order: s^4U, an unknown, 2-methylthio-6-isopentenyl-adenosine (ms^2i^6A), 2-thiocytidine (s^2C), and 5-methylaminomethyl 2-thiouridine (s^2U^*).*

ated according to Abrell et al. (1). Each extract was assayed alone and with added purified factor A or C, obtained from *E. coli* B (1). The extract from the mutant RJ-1 had less than 5% of the parental (NC32) activity, and full activity could be restored by the addition of exogenous factor

TABLE 3. *Activity of the s^4U-forming system in extracts from control and mutant cells*[a]

Enzyme	s^4U-forming activity (U/mg of protein)	
	NC32 (parent)	RJ-1 (mutant)
Purified cell extract	54	3
+ Factor A	79	74
+ Factor C	45	7
Equal amounts of extracts from each strain	38	

[a] Extracts were prepared and assayed as described in the text. All activities were normalized to 1 mg of protein, determined by a modified Lowry et al. method (9). Factor A was added as 16 U of an enzyme prepared from *E. coli* B, purified through the cellulose phosphate column stage (1), 141 U/mg of protein. The factor C supplement, 16 U, was the cellulose phosphate effluent from the same source, 79 U/mg of protein. Neither supplement showed activity by itself.

A (Table 3). Supplementation with exogenous factor C stimulated thiolation somewhat, but the final level of activity in the presence of added factor C was still only 16% of that of the control. Mixture of the RJ-1 and NC32 extracts in equal amounts gave an intermediate value, indicating that the RJ-1 extract does not contain an inhibitor.

Genetic loci governing factors A and C. The gross chromosomal location of genes affecting the activity of the two thiolation enzymes was determined by using F-prime merodiploids. For this procedure, strains of *E. coli* K-12 were obtained which contain duplicated portions of the *E. coli* chromosome as episomes. The genes present on these episomes are probably translated under the same conditions as the genes on the chromosome (8), and thus these gene products are theoretically present in the cell in double amounts. Our set of F-prime episomes included genetic material representing nearly the whole *E. coli* K-12 chromosome, and extracts of cells from each F-prime strain were assayed for activities of factors A and C. Activities were compared with those found in wild-type *E. coli* K-12 grown and assayed under the same conditions. In a total of 19 F-prime strains examined, we found 1 strain which showed increased activity of factor A and 3 with elevated factor C activity. Figures 2 and 3 indicate the portion of the chromosome carried by each of the F-prime episomes concerned with these elevated activities. Table 4 shows the activities found for factors A and C in these strains relative to KL96. Also listed are the activities of these enzymes in

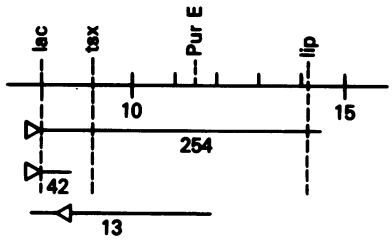

FIG. 2. *Portions of the E. coli K-12 chromosomal map, showing the extent of the genetic material carried on the episome in the F-prime strains exhibiting increased factor A activity. The genetic map is taken from Bachmann et al. (2), and the approximate extent of the F-prime factors is from Low (8).*

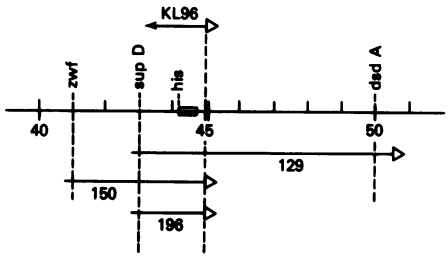

FIG. 3. *The chromosomal regions duplicated on the episome in F-prime strains showing increased factor C activity. The box on the chromosomal map at his, 44 min, indicates the deletion in E. coli strain RW-84 (see text).*

the remaining 14 F-prime strains, where the episomes contained genetic material from other areas of the chromosome (8). None of these activities was markedly different from those of the control.

Episome F254, associated with factor A activity, is large, but certain areas can be excluded. For example, F42 (Fig. 2) includes some of the F254 genes, but it had no additional factor A activity. Similarly, F13 (Fig. 2) was found by other means (see below) to contain a gene for factor A activity. With respect to the factor C region, additional information was furnished by *E. coli* RW-84. This strain had normal levels of factors A and C and tRNA s^4U (data not given). The strain is not a merodiploid, but contains a chromosomal deletion marked in Fig. 3 by a box in the *his* region. Therefore, this deleted region is not involved in s^4U formation.

An attempt was made to normalize the s^4U content of the tRNA in RJ-1 by introducing appropriate episomes. Matings were carried out between RJ-1-4 (which is *lac* negative) and *E. coli* strains carrying F13 or F254. Both F-primes are lac^+ in *lac* negative hosts (see Fig. 2 for maps of the F-primes). Log-phase cultures of RJ-1-4 in M9 minimal medium (2 ml) were

TABLE 4. *Assays of F-prime strains for gene dosage effects in the s^4U-forming system*[a]

F-prime	No. of determinations	Relative sp act[b]	
		Factor A	Factor C
254	4	1.32 ± 0.11	0.92 ± 0.09
42	2	0.08	0.8
13[c]	2	0.07	0.8
129	3	0.83 ± 0.27	1.73 ± 0.25
150	3	0.78 ± 0.16	1.87 ± 0.40
196	3	1.09 ± 0.03	1.49 ± 0.06
14 others[d]	30	0.91 ± 0.17	0.88 ± 0.15

[a] Cells were grown, extracted, and assayed as described in the text. Results are expressed as thiolation units per milligram of protein for the test strain divided by the corresponding activity determined in a parallel culture of *E. coli* strain KL96. Data on the extent of each F-prime factor may be found in reference 8.

[b] Plus or minus standard error of the mean.

[c] This strain is host deleted for the F-prime region (see text).

[d] Includes the following F-prime factors: 104, 111, 112, 116, 117, 123, 126, 128, 133, 140, 141, 142, 143, and 148 (Tables 1 and 2).

mixed with 0.5 ml of log-phase cultures of the F-prime donor, which had been grown in M9 medium supplemented as needed to satisfy the multiple auxotrophies in the host strains. The mating was allowed to proceed for 1.5 h at 25°C without shaking, after which the cells were diluted and plated on minimal agar containing lactose as the sole carbon source, to select for the episomal lac^+ and exclude the donor amino acid auxotrophs. Single-colony isolates were tested for lack of growth at 42°C, since the recipient, RJ-1-4, contains *valS*(Ts). Approximately 10% of the plated cells grew on this medium, and, of these, about half also proved to be temperature sensitive, presumably at the *valS* locus in RJ-1-4. The tRNA's of the progeny were isolated as described in Fig. 1, and the spectra were examined. Normal *E. coli* tRNA has an A_{335} of 1.5 to 1.8% of the A_{260}. Six separate cultures of RJ-1-4 gave a value of 0.13 ± 0.05%, 14 separate isolates from (RJ-1-4 × F13) measured 0.44 ± 0.12% ($P < 0.01$), and 6 separate isolates from (RJ-1-4 × F254) measured 0.48 ± 0.11% ($P < 0.01$ versus RJ-1-4). The values found for the progeny were significantly higher than those for RJ-1-4, although they were still far below normal wild-type levels. It may be that the normal regulation of factor A activity is complex, although it is more likely here that the gene in the episome is under some abnormal regulatory control, possibly even that governing the *lac* operon.

DISCUSSION

Thomas and Favre (15), in their s^4U^- mutant, mapped *nuv*, as the site has been named, at 9.3 ± 0.2 min. This lies within the genetic segments included in F254 and F13 which we have found to contain a gene for factor A activity. It is therefore possible that *nuv* is the structural gene for the thiolation factor A, although, as Thomas and Favre suggest, it may also be a regulatory gene.

If the development of this type of UV resistance requires the absence of s^4U, it could be expected that similar UV-resistant phenotypes might result from a deficiency in the other thiolation enzyme, factor C. However, it has been observed that yeast, which contains neither s^4U nor factor A activity, does have high levels of factor C activity which can complement purified *E. coli* factor A to support normal thiolation (E. E. Kaufman and M. N. Lipsett, unpublished data). Since the total amount of sulfur in yeast tRNA is miniscule, it is likely that this enzyme has some additional role in the cell. If such should also be the case for *E. coli*, the production of the s^4U^- phenotype by factor C deficiency would be possible only if this second function for factor C were dispensable.

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LITERATURE CITED

1. **Abrell, J. W., E. E. Kaufman, and M. N. Lipsett.** 1971. The biosynthesis of 4-thiouridylate. Separation and purification of two enzymes in the transfer ribonucleic acid-sulfurtransferase system. J. Biol. Chem. **246**:294-301.
2. **Bachmann, B. J., K. B. Low, and A. L. Taylor.** 1976. Recalibrated linkage map of *Escherichia coli* K-12. Bacteriol. Rev. **40**:116-167.
3. **Bray, G. A.** 1960. A simple efficient liquid scintillator for counting aqueous solutions in a liquid scintillation counter. Anal. Biochem. **1**:279-285.
4. **Hirota, Y., and P. H. A. Sneath.** 1961. F′ and F-mediated transduction in *Escherichia coli* K-12. Jpn. J. Genet. **36**:307-318.
5. **Hoffman, E. P., and R. C. Wilhelm.** 1970. Genetic mapping and dominance of the amber suppressor, *Su1* (*supD*) in *Escherichia coli* K-12. J. Bacteriol. **103**:32-36.
6. **Lipsett, M. N.** 1965. The isolation of 4-thiouridylic acid from the soluble ribonucleic acid of *Escherichia coli*. J. Biol. Chem. **240**:3975-3978.
7. **Low, B.** 1973. Rapid mapping of conditional and auxotrophic mutants of *Escherichia coli* K-12. J. Bacteriol. **113**:798-812.
8. **Low, K. B.** 1972. *Escherichia coli* K-12 F-prime factors, old and new. Bacteriol. Rev. **36**:587-607.
9. **Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall.** 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. **193**:265-275.
10. **Miller, J. H.** 1972. Experiments in molecular genetics, p. 431. Cold Spring Harbor Laboratory, Cold Spring Harbor, New York.
11. **Peterkofsky, A., and M. N. Lipsett.** 1965. The origin of the sulfur in s-RNA. Biochem. Biophys. Res. Commun. **20**:780-786.
12. **Prasada Rao, Y. S., and J. D. Cherayil.** 1973. Separation of ^{35}S-labelled thionucleosides of *Escherichia coli* and *Pseudomonas aeruginosa* transfer RNA on a phosphocellulose column. Biochim. Biophys. Acta **299**:1-7.
13. **Ramabhadran, T. V., T. Fossum, and J. Jagger.** 1976. *Escherichia coli* mutant lacking 4-thiouridine in its transfer ribonucleic acid. J. Bacteriol. **128**:671-672.
14. **Thomas, G., and A. Favre.** 1977. Mutants de *Escherichia coli* déficients en 4-thiouridine dont la croissance est insensible à l'illumunation à 365 nm. C. R. Acad. Sci. Ser. D **284**:1345-1347.
15. **Thomas, G., and A. Favre.** 1977. Localisation génétique d'une mutation qui rend la croissance de *Escherichia coli* K-12 insensible à l'illumination à 365 nm. C. R. Acad. Sci. Ser. D **284**:2285-2288.
16. **Wolf, R. E., Jr., and D. G. Fraenkel.** 1974. Isolation of specialized transducing bacteriophages for gluconate 6-phosphate dehydrogenase (*gnd*) of *Escherichia coli*. J. Bacteriol. **117**:468-476.