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Characterization of an *E. coli* Mutant with a Thermolabile Initiation Factor IF3 Activity

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Summary. A thermosensitive *E. coli* mutant is described which has at least two defects in vitro: a thermolabile initiation factor IF3 activity and a modified L-phenylalanine: $tRNA^{Phe}$ ligase (EC 6.1.1.20) activity. These two defects cotransduce and are located near 38 min on the new *E. coli* map. Thermoresistant revertants showing in vitro reversion for one defect also revert in vitro for the other defect. The thermosensitive mutation is recessive to its wild type allele, and in vitro analysis of wild type/mutant heterodiploïds also show reversion for both defects.

Introduction

The components participating in the initiation of translation in *E. coli* are now well characterized (Kurland, 1972; Grunberg-Manago et al., 1973; Haselkorn and Rothman-Denes, 1973) and the whole mechanism of the machinery is under intensive study (Shine and Dalgarno, 1975; Argetsinger Steitz and Jakes, 1975; Dahlberg and Dahlberg, 1975; Van Duin et al., 1976). Initiation factors, IF 1, IF 2, and IF 3 belong to the apparently necessary components of this machinery. Initiation factor IF 3 dissociates 70S ribosomes into 30S and 50S subunits (Kaempfer, 1972; Gottlieb et al., 1975), the existence of free 30S subunits being a prerequisite for initiation (Guthrie and Nomura, 1968; Kaempfer et al., 1968). IF 3[1] is also essential for the specific recognition of initiation regions of the mRNA (Revel, 1972). Furthermore, several regulation mechanisms were postulated to be associated with IF 3 action, alone or in conjunction with other components of the initiation system (Lee Huang and Ochoa, 1971; Revel, 1972). These regulatory effects were suggested to occur at the level of discrimination between different species of mRNA and between cistrons in the same RNA. This multiple participation of IF 3 may be explained by a single molecular event at some central locus of the machinery or by multiple interactions at several steps of the initiation. Biochemical experiments have so far failed to answer this fundamental question; thus, we have begun a genetic study of protein synthesis initiation.

In the present work, we describe a mutant which shows thermosensitive IF 3 activity. The same mutant also shows a defect in L-phenylalanine: $tRNA^{Phe}$ ligase (PRL) (EC 6.1.1.20) activity. These two defects could not be separated by transduction or revertant analysis.

Material and Methods

Strains. The relevant characteristics of the strains used in this work are given in Table 1.

Genetic Procedures. The thermosensitive mutant C 18 was isolated in the laboratory of Dr. G. Tocchini-Valentini by the tritiated amino acid "suicide" procedure (Tocchini-Valentini et al., 1969). Classical methods were employed to map the thermosensitive mutation (Low, 1973; Miller, 1972). Transductions using *P1 vir.* were performed as described by Miller (1972). *rec A* derivatives were constructed by first selecting for *thy A* with trimethoprim and then for *thy* A^+ *rec A* recombinants by conjugation with a *rec A thy* A^+ Hfr (Miller, 1972).

Cell Growth. Strains were grown at 30° C in Difco antibiotic medium n° 3 to 1 A_{650}, cooled, centrifuged, washed and stored at −20° C. Thermosensitive strains were checked as having a low proportion of thermoresistant (growth at 42° C) revertants. Diploid strains were checked for the presence of the episome by comparing their plating efficiencies on rich and appropriate minimal plates (where only diploids grow); the heterodiploid strain AB 1500 KLF 48 was also checked for the presence of mutant allele after growth, by reappearance of the thermosensitive phenotype after loss of the episome. The loss of the episome KLF 48 was induced by selecting for the presence of another episome (KLF 10 covering *argE*).

Extracts. Cells were ground with twice their weight of alumina and homogenized with 1.5 ml of grinding buffer per gram of cells;

[1] Abreviations: IFX = initiation factor X. PRL = L-phenylalanine: $tRNA^{Phe}$ ligase (EC 6.1.1.20). fMet-tRNA = formyl-methionyl-$tRNA^{Met}$

Table 1. *E. coli* strains

Strains	Relevant genotype		Origin
D 1	Hfr C *met*$^-$		G.P. Tocchini-Valentini
C 18	Hfr C *met*$^-$ *ts*$^-$ *C18*		G.P. Tocchini-Valentini
AB 1360	F$^-$ *argE3 his4 proA2 thi galK2 lacY1 aroD6*		M. Hoffnung
AB 1361	F$^-$ *argE3 his4 proA2 thi galK2 lacY1 ts*$^-$ *C18*		Thermosensitive transductant of AB 1360 (P1vir grown on C 18)
R AB 1, 3, 7, 8	F$^-$ *argE3 his4 proA2 thi galK2 lacY1*		Thermoresistant spontaneous revertants of AB 1361
AB 1365	F$^-$ *argE3 his4 proA2 thi galK2 lacY1*		Thermoresistant transductant of AB 1360 (P1vir. grown on C 18)
AB 1500	F$^-$ *argE3 his4 proA2 thi galK2 lacY1 ts*$^-$ *C18 recA rif*		*recA rif* derivative of AB 1361
AB 1400	F$^-$ *argE3 his4 proA2 thi galK2 lacY1 aroD6 recA rif*		*recA rif* derivative of 1360
AB 1500 KLF 48	chromosome: episome:	AB 1500 KLF 48 carries *his* to *aroD* and a *cheB-cheC* deletion	episome from B. Bachmann E. coli genetic stock center
AB 1400 KLF 48	chromosome: episome:	AB 1400 KLF 48	

the grinding buffer was as follows: Tris HCl pH 7.5, 10 mM; NH_4Cl, 60 mM; Mg acetate, 10 mM; β-mercaptoethanol, 7 mM; glycerol 10%. After low speed centrifugation (30 min at 30,000 g) the supernatant (S 30) was centrifuged for $2^1/_2$ h at 50,000 RPM in a Spinco 65 rotor. The ribosomal pellet was resuspended in the washing buffer (Tris HCl pH 7.5, 10 mM; NH_4Cl, 1.5 M; Mg acetate, 10 mM; β-mercaptoethanol, 7 mM) and centrifuged again under the same conditions. The crude IF fraction was obtained from the supernatant of this last centrifugation after dialysis against Tβ buffer (Tris HCl pH 7.5, 10 mM; β-mercaptoethanol, 7 mM). The fraction called SA2 was obtained from the 55 to 75% ammonium sulphate fraction of the crude IF fraction after dialysis against Tβ buffer. DEAE eluates were obtained from the first high speed centrifugation supernatant as described by Muench and Berg (1966), concentrated in 5 mM phosphate buffer pH 6.8 with 20 mM β-mercaptoethanol and 50% glycerol and kept at $-20°$ C. Protein concentrations were measured by the method of Lowry et al. (1951) with beef serum albumin as standard.

Reagents. Ribosomes used in the different tests were purified from MRE 600 as described above under "extracts" except that high speed centrifugation in the presence of washing buffer was preceded by two and followed by one, high speed centrifugations in grinding buffer without glycerol. The supernatant (S 150) used in the phenylalanine incorporation test was made from the upper 2/3 fraction of the supernatant from the first high speed centrifugation and dialyzed against 10 mM Tris HCl pH 7.5 and 7 mM β-mercaptoethanol. IF 1, IF 2, and IF 3 were prepared from MRE 600 as already described (Dondon et al., 1974). [^{3}H]fMet-tRNA and random poly (A, U, G) were prepared as described elsewhere (Lelong et al., 1970). [^{3}H] and [^{14}C] amino acids were from CEA (France). Other reagents were of highest commercial quality available.

Results

Genetical Characterization

The *E. coli* mutant C 18 was obtained from strain D1 after nitrosoguanidine mutagenesis and enrichment for thermosensitive mutants with the tritiated amino acid "suicide" method (Tocchini-Valentini et al., 1969). The thermosensitive mutation of C 18 (*ts*$^-$ *C18*) is located near 38 min on the new *E. coli* map (Bachmann et al., 1976) and is 68% co-transducible with the *aro D* marker. The reversion frequency to thermoresistance of C 18 is approximately 10^{-8}, identical to that of AB 1361 (a thermosensitive transductant which is also *ts*$^-$ *C 18*). This suggests that a single mutation is responsible for the thermosensitive phenotype in both C 18 and AB 1361. AB 1361 was made *recA*$^-$, and then diploid for the 38 min region using the episome KLF 48. The stable heterodiploid AB 1500 KLF 48 thus constructed was shown to be thermoresistant. The presence of the episome in this thermoresistant heterodiploid was verified by the auxotrophic properties of the strain and by its capacity of transferring KLF 48 to a female strain with high frequency; the presence of the thermosensitive allele in this same strain was verified by reappearance of the thermosensitive phenotype after loss of the episome KLF 48. The thermosensitive mutation of C 18 (and AB 1361) is thus recessive to its wild-type allele.

Characterization of the Initiation Factor IF 3 Activity Defect

IF 3 was tested for three different activities: stimulation of fMet-tRNA binding to ribosomes in the presence of poly (A, U, G); dissociation of 70S ribosomes into 50S and 30S subunits; stimulation of phenylala-

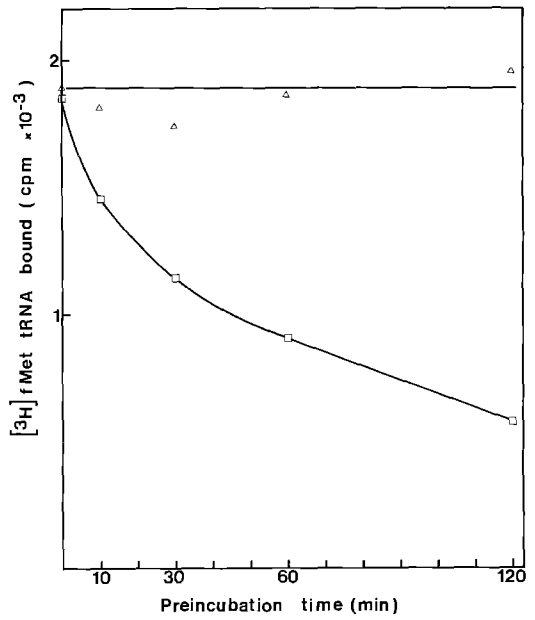

Fig. 1. Inactivation kinetics of crude IF fractions from mutant and parental strains. Preincubation was carried out at 43° C. The preincubation mixture contained: Tris HCl pH 7.5, 50 mM; NH_4Cl, 50 mM; Mg acetate, 5 mM (TMN buffer); crude IF fraction from C 18 (0.49 mg/ml) or crude IF fraction from D 1 (0.32 mg/ml). Total protein concentration in the preincubation was brought to 1 mg/ml with beef serum albumine (BSA). At times indicated in the figure, 25 µl aliquots were withdrawn, chilled to 0° C, and completed to 100 µl with the incubation mixtures. This incubation mixture (100 µl) contained besides preincubated proteins: TMN buffer; GTP, 0.13 µmoles; ribosomes, 1.9 A_{260}; [^{3}H]fMet-tRNA, 0.5 A_{260} (14.3 pmole/A_{260}); poly (A, U, G), 0.16 A_{260}. Incubation was for 15 min at 37°. The samples are then diluted with 2 ml of cold TMN buffer. The samples were filtered at once on a Millipore nitrocellulose membrane (HAWP 02500). The filters were rinced twice with 3 ml of TMN buffer, dried, and counted. These conditions yielded 2500 CPM per pmole of [^{3}H]fMet-tRNA. Blanks without poly (A, U, G) and without crude IF fraction were substracted. □, crude IF fraction from C 18; △, crude IF fraction from D 1

nine incorporation into polyphenylalanine in the presence of poly (U) at 18 mM Mg^{2+} (Schiff et al., 1974). The original thermosensitive strain C 18 was always compared with the parental strain D 1, and the thermosensitive transductant AB 1361 with the thermoresistant transductant AB 1365.

When preincubated at 43° C the 1.5 M ammonium chloride ribosomal wash (crude IF fraction) of strain C 18 shows inactivation kinetics different from those of strain D 1 (Fig. 1). fMet-tRNA binding capacity of the mutant crude IF fraction is 36% of its original value after two hours of preincubation, whereas the activity of the parental strain is stable. IF 3 thermal inactivation in this test and the following is measured in the zone where activity is linearly dependent on the amount of proteins added. Furthermore, the different heat inactivation curves of wild-type and mutant crude IF fractions cannot be explained by the appearance of an inhibitor or by the action of a protease during preincubation. In fact mixtures of wild-type and mutant fractions in limiting amounts show intermediary inactivations corresponding to the proportions of each fraction added during preincubation (data not shown). It is known that the fMet-tRNA binding to ribosomes in the presence of poly (A, U, G) is totally dependent on initiation factor IF 2, but only stimulated by IF 1 and IF 3 (Dondon et al., 1974). The poly (A, U, G) dependent fMet-tRNA binding test is therefore not sufficient to characterize the thermolabile activity and is poor test to study a possible defect in IF 1 or IF 3 activity.

Dissociation of 70S ribosomes into 30S and 50S subunits is dependent on IF 3, stimulated by IF 1, and somewhat inhibited by IF 2 (Godefroy-Colburn et al., 1975). Preincubation of the crude IF fraction at 42° C during 30 min almost completely abolishes the dissociation activity in strain C 18, whereas the inactivation in the parental strain D 1 is only partial (Fig. 2). The fact that preincubation at relatively low temperatures also affects the dissociation activity of the crude IF fraction of the wild-type strain may seem in contradiction with the known heat stability of IF 3, but was also observed previously (Kaempfer, 1972). This relatively low heat stability of wild-type dissociation activity (and other IF 3 activities) in crude IF fractions was not investigated since only the differences between wild-type and mutant activities at the same purification step were examined.

As stated above, dissociation activity is not quite specific for IF 3 and therefore in vitro complementation of the defective activity was studied with pure initiation factors. In the fMet-tRNA binding test, the presence of pure IF 3 from the wild-type strain in the preincubation mixture eliminates the thermolability of the crude IF fraction from C 18; under the same conditions IF 1 does not eliminate this thermolability (Table 2). The addition of IF 2 has a somewhat intermediary effect. Such an effect could be expected from an IF 3 defect since it is known that at high IF 2 concentrations fMet-tRNA binding in the presence of poly (A, U, G) is poorly stimulated by IF 3 (Dondon et al., 1974) which means that the addition of exogeneous IF 2 lowers the IF 3 mediated stimulation of the test and thus also the measured thermolability of IF 3 activity. The 55–75% ammonium sulphate fraction of the ribosomal ammonium chloride wash (SA2) of AB 1361 (thermosensitive transductant) which contains IF 3 is also thermolabile and is also complemented with pure IF 3 (Table 2). In these experiments the purified initiation factors were added in excess as compared to their quantities in the crude IF or SA2 fractions.

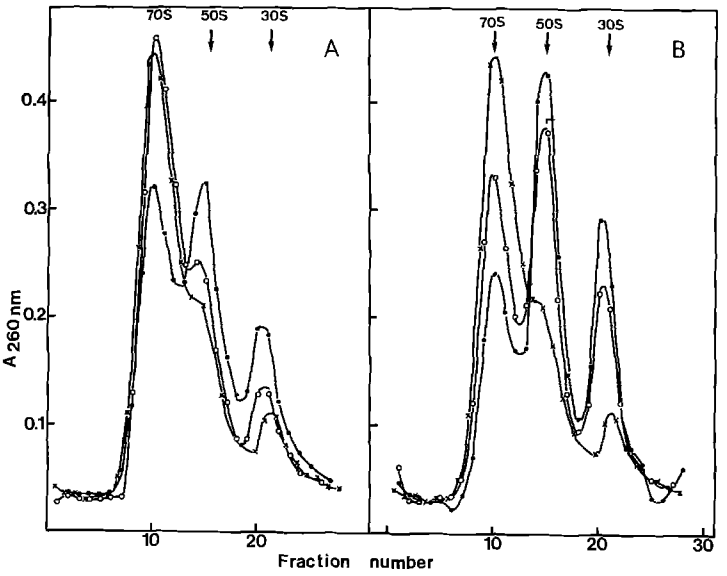

Fig. 2A and B. Inactivation of dissociation in crude IF fraction from mutant and parental strains. Preincubation was carried out at 42° C during 30 min in 100 µl containing TMN buffer and 0.1 mg of crude IF fractions. After preincubation the samples were chilled and 1.9 A_{260} of ribosomes in 20 µl of TMN buffer were added per sample for further incubation. After 30 min at 37° C the samples were chilled again and 100 µl were loaded on the top of a 10 to 30% sucrose gradient in TMN buffer. Centrifugation was carried out a 4° C in a Spinco SW56 rotor for 2 h at 50,000 RPM. The gradient was collected from the bottom part of the tube. The 120 µl fractions were diluted with 300 µl of water and absorbancy was measured at 260 nm. **A.** ×, ribosomes incubated alone; •, ribosomes incubated with crude IF fraction from C18; ○, ribosomes incubated with preincubated crude IF fraction from C18. **B.** ×, ribosomes incubated alone; •, ribosomes incubated with crude IF fraction from D1; ○, ribosomes incubated with preincubated crude IF fraction from D1

Table 2. Complementation with purified initiation factors

Additions		Binding of fMet-tRNA to ribosomes (CPM)					
		mutant			wild-type		
		Preincubation		Activity after preincubation (%)	Preincubation		Activity after preincubation (%)
		−	+		−	+	
Crude IF fraction		2411	1385	57	2913	3517	121
+IF 1		2332	1113	48	3384	3437	102
+IF 2		5743	4188	73	5214	4992	96
+IF 3		5861	5706	97	3579	4058	113
55–75%	*	2369	624	26	* 2727	2226	81
$SO_4(NH_4)_2$ (SA 2)	†	5240	1252	23	† 6317	5866	92
+IF 3	*	3147	2679	85	* 2646	2147	81
	†	6677	5725	85	† 5309	4506	84

In the experiment with crude IF fractions preincubation was carried out at 42° C for 30 min and the preincubation mixture (25 µl) contained: TMN buffer, crude IF fraction of C 18, 17.5 µg or crude IF fraction from D 1, 27.5 µg. When present IF 1 was at 10 µg/ml, IF 2 at 20 µg/ml and IF 3 at 22 µg/ml. The protein concentration in the preincubation mixture was brought to 1 mg/ml with BSA. For incubation the samples were then processed as in Figure 1. Blanks without poly (A, U, G) and without crude IF fractions were substracted. 1 pmole of [^{3}H]fMet-tRNA yielded 2500 CPM

In the experiment with SA 2 fractions, preincubation was carried out at 50° C for 30 min and the preincubation mixture contained: TMN buffer; SA 2 from AB 1361, 0.48 mg/ml or SA 2 from AB 1365, 0.4 mg/ml. When IF 3 (5 µg/ml) was present SA 2 from AB 1361 was at 0.24 mg/ml and SA 2 from AB 1365 was at 0.20 mg/ml. The final protein concentration in the preincubation mixture was brought to 0.5 mg/ml with BSA. After preincubation 25 µl (*) and 50 µl (†) aliquot were chilled and completed to 100 µl with the same incubation mixture as in Figure 1 except that IF 2 was added at 0.75 µg per sample, and that [^{3}H]fMet-tRNA (2.8 pmoles/A_{260}) was added at 2.7 A_{260} per sample. Incubation was at 37° C for 15 min. The processing of the samples was the same as in Figure 1. 1 pmole of [^{3}H]fMet-tRNA yielded 4200 CPM

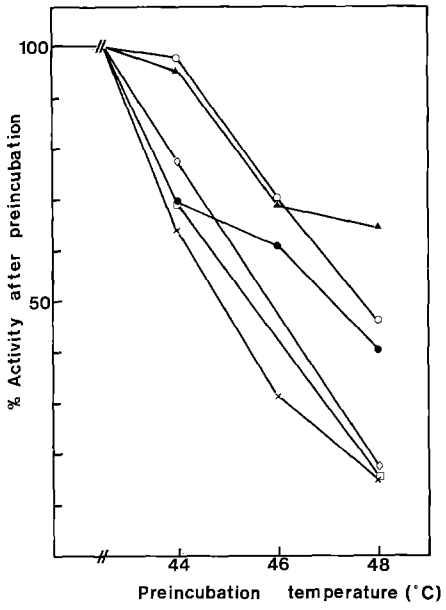

Fig. 3. Thermal inactivation of IF 3 activity measured with the phenylalanine incorporation test. Preincubation was carried out for 30 min at the indicated temperatures in TMN buffer with the following SA 2 concentrations: 0.45 mg/ml for AB 1361, 0.20 mg/ml for RAB 1, 0.6 mg/ml for RAB 3, 0.22 mg/ml for RAB 7, 0.50 mg/ml for RAB 8, 0.40 mg/ml for AB 1365. The final protein concentration in the preincubation mixture was brought to 0.5 mg/ml with BSA. After preincubation 50 µl aliquots were chilled and completed to 100 µl for incubation. Incubation mixture (100 µl) contained: Tris HCl pH 7.5, 60 mM; NH_4Cl, 100 mM; Mg acetate, 18 mM, β-mercaptoethanol, 14 mM; ATP, 1.5 mM; GTP, 0.5 mM; creatine phosphate, 1.5 mM; [^{14}C]phenylalanine (10mCi/mmole), 0.1 mM, 19 other aminoacids, 0.1 mM; tRNA, 1.5 A_{260}; poly U, 0.07 A_{260}; creatine phosphokinase, 8 µg; ribosomes, 5 A_{260}; S 150 supernatant, 0.1 mg; SA 2 fractions at half the concentration of preincubation. After incubation at 37° C for 20 min, 1.5 ml of NaCl 1 M and 1.5 ml of 10% TCA were added. The samples were again incubated 15 min at 85° C, the precipitate, collected on Whatmann GF/C filters, was washed twice with 1% TCA, dried and counted. The PRL activity of the SA 2 fractions was negligible compared to the activity added with the S 150 supernatant in the incubation mixture. The 100% point corresponds to 2572 CPM for AB 1361, 2240 for RAB 1, 2725 for RAB 3, 2229 for RAB 7, 2418 for RAB 8 and 2468 for AB 1365. For these values blanks without SA 2 (1500 CPM) are substracted. Blanks without poly U are negligible. Under the present conditions 1 pmole of phenylalanine yielded 20 CPM. ×, SA 2 from AB 1361; ●, SA 2 from RAB 1; □, SA 2 from RAB 3; ○, SA 2 from RAB 7; ◇, SA 2 from RAB 8; ▲, SA 2 from AB 1365

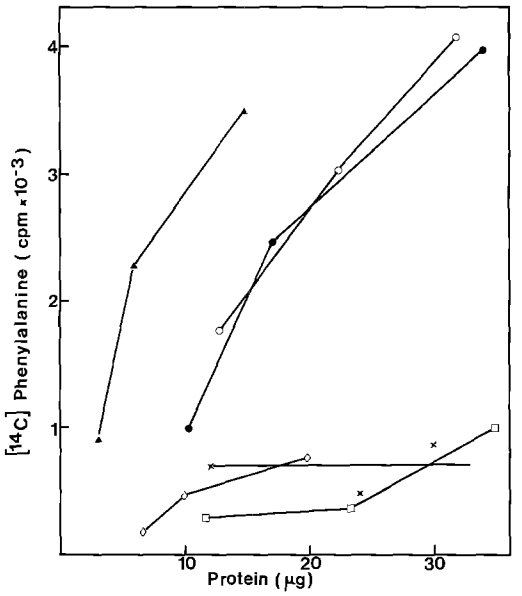

Fig. 4. PRL activity in mutant, revertant and wild-type strains. Incubation mixtures contained (100 µl): Tris HCl pH 7.5; 100 mM; Mg acetate, 10 mM; β-mercaptoethanol, 7 mM; ATP, 1 mM; tRNA, 5 A_{260}; [^{14}C]phenylalanine, 2.3 m µmoles; 19 other aminoacids, 0.02 mM; glycerol, 15%; DEAE eluates as indicated. After 10 min at 37° C, the samples were diluted with 2 ml of cold 5% TCA with 0.2% casamino acids and the precipitate collected on GF/C Whatman filters which were washed twice with 4 ml of cold 1% TCA with 0.2% casamino acids, rinced once with 4 ml ethanol, dried, and counted. Under these conditions 1 pmole of phenylalanine yielded 270 CPM. Blanks without added tRNA are substracted (these are inferior to 10% of the counts in the presence of added tRNA) ×, AB 1361; ●, RAB 1; □, RAB 3; ○, RAB 7; ◇, RAB 8; ▲, AB 1365

The thermolability of the dissociation activity of the crude IF fraction of C 18 (compared to D 1), and the in vitro complementation of the thermolabile defect with pure IF 3 and not with IF 1, suggest a defect in IF 3 activity. In order to unambiguously characterize the modified activity, the stimulation of incorporation of phenylalanine into polyphenylalanine in the presence of poly (U) at 18 mM Mg^{2+} was used as specific test for IF 3 since IF 1 and IF 2 are known to be without effect (Schiff et al., 1974).

A 30 min preincubation at 44° C, 46° C and 48° C differently affects the SA2 fraction of AB 1361 and of AB 1365 (Fig. 3). In this test also, addition of SA2 of AB 1365 to the SA2 of AB 1361 reverses thermolability according to the proportion of extracts preincubated together (data not shown).

Characterization of the L-phenylalanine: tRNA^{Phe} Ligase Activity Defect

C 18 was isolated with the tritiated amino acid method which is known to yield many amino acid "suicide" tRNA ligase mutants (Tocchini-Valentini, personal communication). The fact that the *ts⁻ C18* mutation is located at about 38 min near *pheS* and *tyrS* prompted us to look for L-phenylalanine:tRNA^{Phe} ligase (PRL) and L-tyrosine:tRNA^{Tyr} ligase (EC 6.1.1.1) activities in the extracts of AB 1361 and AB 1365. Figure 4 shows that the DEAE-cellulose eluate of a ribosomal supernatant of AB 1361 has very little PRL activity. Contrarywise, in the same

extract, L-tyrosine:tRNA^{Tyr} ligase, L-Methionine:tRNA^{Met} ligase (EC 1.1.10), and L-valine:tRNA^{Val} ligase (EC 6.1.1.9) activities are quite similar to wild-type activities (data not shown).

The low residual PRL activity in AB 1361 cannot be explained by a specific inhibitor since when this AB 1361 extract was added to the AB 1365 extract, PRL activity was not inhibited (data not shown). In the DEAE extracts of C 18 the same low PRL activity was observed whereas extracts of the parental strain D1 were found to be normally active.

Analysis of Spontaneous Thermoresistant Revertants

The above data show that the defects in IF 3 and in PRL activity are present in both C 18 and AB 1361, and thus do cotransduce. The same two defective activities were characterized in four independent thermosensitive transductants. Since these defects could not easily be separated by transduction, four fast growing independent spontaneous thermoresistant revertants of AB 1361 were analyzed in order to find out whether one of the two defects could possibly revert alone. The thermolability of the IF 3 activity was studied in the revertant strains using the IF 3 specific phenylalanine incorporation test. The SA2 fractions of RAB 1 and RAB 7 show an intermediary thermolability between wild-type (AB 1365) and mutant (AB 1361) strains (Fig. 3). On the contrary, two other revertants RAB 3 and RAB 8 show an IF 3 activity as thermolabile as that of the mutant strain (AB 1361). The same reversion pattern in vitro is seen with the fMet-tRNA binding test (data not shown).

The same revertants were then analyzed for their PRL activity. Figure 4 shows that the PRL activity reversion pattern is analogous to that of IF 3 thermolability. The first group of revertants (RAB 1 and RAB 7) which showed an intermediary IF 3 thermolability are characterized by a PRL activity that is also intermediary. The second group of revertants (RAB 3 and RAB 8) which showed no in vitro reversion of the IF 3 thermolability show only a low residual PRL activity. No revertant was found showing reversion of an activity without the other. The comparatively low PRL activities of RAB 1 and RAB 7 extracts cannot be explained by specific inhibitors because these fractions are normally complemented by wild-type fractions or pure PRL. The residual activities of the DEAE eluates from the revertant strains RAB 1 and RAB 7 were checked as being true PRL activities by addition of [^{12}C]phenylalanine in the incubation mixture, which resulted a loss of TCA precipitable counts (data not shown).

The low PRL activity in the extracts of AB 1361 can be explained either by a low quantity of normal PRL or by some structural modification of the PRL. A structural defect in the mutant can be shown by a thermolability difference between mutant and wild-type activities. The thermolability of PRL activity cannot be studied with AB 1361 because of its low residual activity; on the contrary, this activity can be studied in extracts of RAB 1 and RAB 7. Figure 5 shows that PRL activity of AB 1365 is more thermostable than that of RAB 1 and of RAB 7. This suggests some structural difference between PRL in AB 1365 and in the revertants RAB 1 and RAB 7; this in turn suggests that low PRL activity in AB 1361 extracts is explained by some structural defect of PRL in that strain.

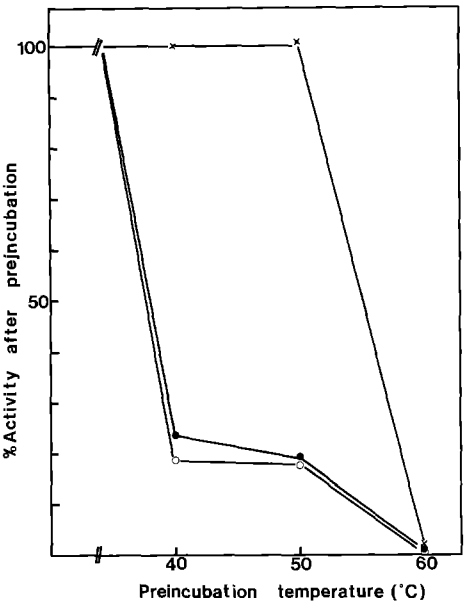

Fig. 5. Thermal inactivation of wild-type and revertant PRL activity. Preincubation was carried out for 30 min at the indicated temperatures. The preincubation mixture contained phosphate buffer pH 6.8, 5 mM; β-mercaptoethanol, 20 mM; glycerol, 50%. DEAE eluates were present at 2.65 mg/ml for RAB 1, 2.61 mg/ml for RAB 7, 0.58 mg/ml for AB 1365. The protein concentration in the preincubation mixture was brought to 2.65 mg/ml with BSA. After preincubation aliquots were chilled and completed to 100 µl with the same incubation mixture as in Figure 4. In the incubation DEAE eluates were present at 0.53 mg/ml for RAB 1; 0.51 mg/ml for RAB 7; 0.1 mg/ml for AB 1365. The samples were processed as in Figure 4. The 100% point corresponds to 7598 CPM for RAB 1, 6544 CPM for RAB 7, 11900 CPM for AB 1365. ×, AB 1365; ●, RAB 1; ○, RAB 7

Diploid Analysis in vitro

The heterodiploid AB 1500 KLF 48 is thermoresistant in vivo. In vitro study of the extracts from this heterodiploïd might tell us wether one or both defects

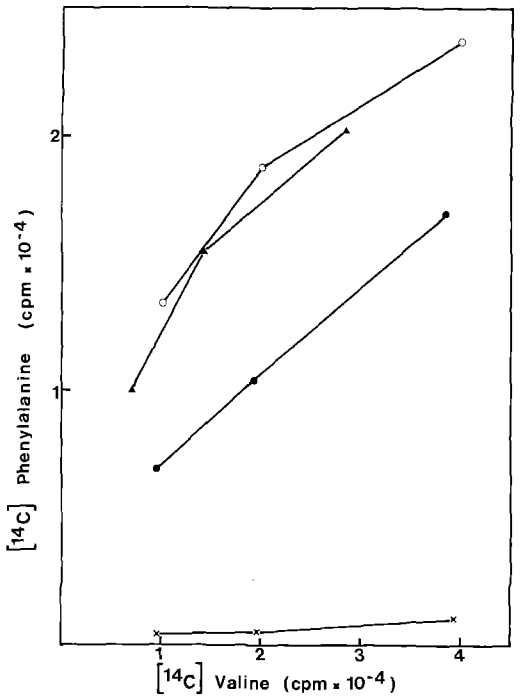

Fig. 6. PRL activity in haploid and diploid strains. Conditions were as in Figure 4, except that glycerol was present at 10% in the incubation mixture. The PRL activity is given in function of valine: $tRNA^{Val}$ ligase activity which was determined under the same conditions as PRL activity except that [^{14}C] valine (concentration 2 m µmole/100 µl, specific activity: 20 µCi/µmole) was used instead of [^{14}C]phenylalanine and that the mixture of amino acids without phenylalanine was not used. Under these conditions (40 CPM per pmole of valine) one has 3850 CPM/µg of DEAE eluate of AB 1500; 3500 CPM/µg for AB 1400; 3900 CPM/µg for AB 1500 KLF 48; and 3700 CPM/µg for AB 1400 KLF 48. ×, AB 1500; ●, AB 1400; ○, AB 1500 KLF 48; ▲, AB 1400 KLF 48

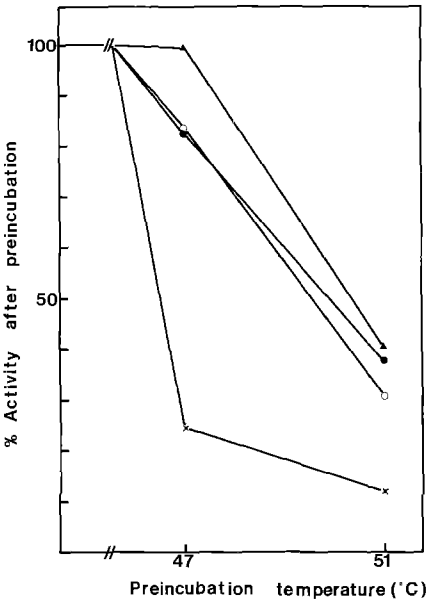

Fig. 7. Thermolability of IF 3 activity in haploid and diploid strains. Preincubation was carried out for 30 min at the indicated temperatures in TMN buffer. In the preincubation the SA 2 concentrations were 0.21 mg/ml for AB 1500, 0.18 mg/ml for AB 1400, 0.12 mg/ml for AB 1500 KLF 48, 0.15 mg/ml for AB 1400 KLF 48. The final protein concentration in the preincubation was brought to 0.2 mg/ml with BSA. After preincubation 25 µl aliquots for AB 1500, 20 µl aliquots for AB 1400 and AB 1500 KLF 48, and 15 µl aliquots for AB 1400 KLF 48 were chilled and completed to 100 µl for incubation. Incubation mixture was the same as in Figure 1 except that IF 2 was added at 0.7 µg per assay and that another preparation fMet-tRNA was used (7.5 pmoles/A_{260}) at 0.5 A_{260} per assay. Incubation was 15 min at 37° C and further processing as in Figure 1. When blanks without SA 2 are substracted, the 100% point corresponds to 3780 CPM for AB 1500, 2950 CPM for AB 1400, 3550 CPM for AB 1500 KLF 48, 3400 CPM for AB 1400 KLF 48. Blank without SA 2: 1400 CPM (3230 CPM/pmole). ×, AB 1500; ●, AB 1400; ○, AB 1500 KLF 48; △, AB 1400 KLF 48

of AB 1500 are reverted. A single reverted defect in the extracts of the heterodiploïd would identify the primary cause of this strains phenotype.

Four strains, the mutant haploid (AB 1500), the wild-type haploid (AB 1400), the wild-type homodiploid (AB 1400 KLF 48) and the heterodiploid (AB 1500 KLF 48) were first compared for PRL activity.

L-valine: $tRNA^{Val}$ ligase activity was first measured in the different strains as a function of the amount of extract added in the test; a proportional relation between L-valine: $tRNA^{Val}$ ligase activity and the amount of protein was found for each strain; this enabled the expression of PRL as a function of valine: $tRNA^{Val}$ ligase activity. The latter was used as control because *valS* (structural gene of L-valine: $tRNA^{Val}$ ligase) is very distant from the 38 min region of the *E. coli* chromosome and is not covered by KLF 48 (Low, 1972). Figure 6 shows that the heterodiploid PRL activity is equal to that of the homodiploid and higher than that of the wild-type haploid. The presence of the mutant allele in the heterodiploid was verified in vivo by the reappearance of the thermosensitive phenotype with loss of KLF 48, and in vitro by showing that PRL activity of the heterodiploid, albeit higher than the activity of the wild-type haploid, is more thermolabile than that of the wild-type haploid and homodiploid (Table 3). The same strains were analyzed for the thermolability of the IF 3 activity in the fMet-tRNA binding test (Fig. 7) and in

Table 3. Thermolability of PRL activity on diploid extracts

		[^{14}C]phenylalanyl-tRNA (CPM)			
		AB 1500	AB 1400	AB 1500 KLF 48	AB 1400 KLF 48
− preincubation	(*)	689	7,233	7,137	6,621
	(†)	1,122	12,564	12,620	11,346
+ preincubation	(*)		5,903	3,477	5,140
	(†)		10,130	6,451	9,685
% activity left	(*)		81	48	77
of the preincubation	(†)		80	51	85

Preincubation was carried out 30 min at 55° C. The preincubation mixture was the same as in Figure 5 except that DEAE eluates were present at 1.02 mg/ml for AB 1500, 0.55 mg/ml for AB 1400, 0.21 mg/ml for AB 1500 KLF 48, 0.185 mg/ml for AB 1400 KLF 48. Protein concentration in the preincubation was brought to 1 mg/ml with BSA. After preincubation 5 (*) and 10 µl (†) aliquots were chilled and completed to 100 µl with the same incubation mixture as in Figure 4 except that the amino acid mixture without phenylalanine was omitted. The samples were then processed as in Figure 4

the stimulation of phenylalanine incorporation (data not shown). The only IF 3 activity found to be clearly thermolabile is that of the mutant haploid. It thus seems that the presence of a wild-type allele in the heterodiploïd reverses both PRL activity and IF 3 thermolability.

Discussion

In the thermosensitive mutants C 18 and AB 1361 (thermosensitive transductant derived from C 18) an IF 3 activity defect was characterized by the thermolability a) of its fMet-tRNA binding activity, b) of its dissociation activity, and c) of its activity in the polyphenylalanine incorporation test. These defects were shown to exist in the crude IF and in the SA 2 fractions. Structural modification of the IF 3 protein itself could not be shown because IF 3 activity in the mutant strain (and only in the mutant strain) is lost before purification to homogeneity. Purification of IF 1 and IF 2 from C 18 (mutant strain) and D 1 (parental strain) showed that thermolability of each of the two factors is identical in both strains.

The same mutant also shows a defect in PRL activity. The low level of PRL activity in the mutant strain is very probably due to a structural defect in the enzyme and not to a very low level of wild-type PRL activity because: a) the revertants RAB 1 and RAB 7 have a measurable PRL activity and this activity is more thermolabile than that of the wild-type (AB 1365), and b) the heterodiploïd also has PRL activity which is more thermolabile than that of the wild-type.

Both defects (PRL activity and IF 3 thermolabile activity) are completely or partially compensated by a) in vitro complementation with pure wild-type molecules; b) in vivo reversion of the thermosensitive phenotype; and c) the presence of a wild-type allele in heterodiploïds.

The isolation of thermoresistant revertants with no observable in vitro reversion for PRL or IF 3 activity might be explained by an external suppression reversing the thermosensitive phenotype without modifying the mutant PRL or IF 3 activities. Several cases of external suppression of thermosensitive mutants modified in amino acid: tRNA ligases have already been reported (Russel and Pittard, 1971; Böck et al., 1974). The existence of revertants in which both IF 3 thermolabile activity and low PRL activity are reverted, indicates some relation or interaction between PRL and IF 3. This relation or interaction could theoretically take place at the genetic level (i.e. IF 3 and PRL would share some common gene); at the transcription level (by some regulatory effect of one molecule on the transcription of the other); or at the translational or post-translational level.

Three facts argue against IF 3 and PRL sharing some common gene:

1. The structural properties of the two proteins are totally different; IF 3 has a molecular weight of 20,000 daltons and PRL, which is a tetramer of $\alpha_2\beta_2$ type, has subunits molecular weights of 38,000 daltons for α and of 96,000 daltons for β (Fayat, 1974; Hanke et al., 1974).

2. No interaction whatsoever could be characterized at the level of activities. Pure IF 3 has no PRL activity and does not stimulate PRL activity in a mutant extract; pure PRL has no IF 3 activity and does not protect IF 3 against heat inactivation.

3. Preliminary experiments revealed no common antigenic determinants between pure IF 3 and pure PRL, using IF 3 and PRL antibodies.

Neither could some relation or interaction between PRL and IF 3 at the transcription level satisfactorily explain the results. If one of the two proteins

regulates the transcription of the other, structural defect in one molecule should lead to a decrease of the level of the other protein and should not, as apparently is the case here, result in a double structural defect. The evidences in favour of a structural defect of PRL in the mutant were already discussed; moreover, the IF 3 defect is characterized by a thermolabile activity which is better explained by some structural modification than by a decreased level of a structurally wild-type protein.

Some interaction or relation at the translational level cannot be ruled out. Mutational modification of a protein involved in translation might well influence translation of other proteins, as does for instance a mutational modification of the stringent factor in starved cells (Hall and Gallant, 1972). Post-translational modification of both IF 3 and PRL by a modifying enzyme altered in C 18 (or AB 1361) might also explain some of the results. In both cases other defective activities should eventually be found in the mutant strains.

In spite of the reversion frequency which is that of a single mutant it is not possible to completely exclude the possibility that the two in vitro reverted defects in spontaneous revertants are a consequence of a double reversion event. It is possible, that a double thermosensitive mutant with one tight and one leaky mutation could yield double revertants at an apparent frequency which is that of a simple mutant. When the tight mutation reverts, the remaining leaky mutation permits growth until a sufficient number of cells are present to give a second reversion. This double event will give thermoresistant colonies with a frequency equal to that of the reversion of the tight mutation, and the double revertants in these colonies will be selected during further purification at high temperature. The in vitro analysis of thermoresistant revertants was done only with fast growing revertants selected at 42° C; slow growing revertants selected at lower temperature are being studied to investigate this possibility.

A bacteriophage λ has recently been isolated (Springer et al., 1976) which transduces the mutant thermosensitive strain AB 1361 to thermoresistance. The bacteriophage complements both IF 3 and PRL activities in vitro, exactly as does the episome KLF 48. The coding properties of this bacteriophage are now being studied to understand the relation between the two defects of the thermosensitive mutant characterized in this study.

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Note Added in Proof

The bacteriophage λ tranducing AB 1361 to thermoresistance was shown to carry the genes for IF3 and for both subunits of PRL which means that theese three structural genes are located close together at 38 min on the *E. coli* chromosome.