IN VITRO STUDIES OF PROTEIN SYNTHESIS IN DROSOPHILA

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Summary

Comparative in vitro studies of protein synthesis in Oregon-R wild-type and A^{53g} mutant Drosophila melanogaster indicate that the A^{53g} genotype has an effect at the translation level. The A^{53g} post-microsomal supernatant fluid stimulates the incorporation of C^{14} amino acids by cell-free systems using ribosomes prepared from either A^{53g} or Oregon-R wild-type flies. The level of in vitro charging of Drosophila and calf liver tRNA with C^{14} amino acids by the A^{53g} post-microsomal supernatant fraction is also greater than the level of charging by the Oregon-R supernatant fluid.

A great deal of information is known about the genetics and developmental biochemistry of Drosophila, but relatively little is known about the genetic control of protein synthesis at the molecular level in this insect. The present paper reports initial in vitro investigations of protein synthesis using the mutant, Abnormal Abdomen (A^{53g}), in Drosophila melanogaster. This genotype, which consists of a sex-linked major gene and a series of sex-linked and autosomal modifiers, causes the production of abnormal abdominal tergites in the adult fly (Hillman, 1953).

The in vitro techniques used are: 1) a cell-free, C^{14} amino acid incorporating system using Drosophila ribosomes, endogenous messenger, and Drosophila supernatant fractions; and 2) in vitro charging of Drosophila transfer RNA (tRNA) and calf liver tRNA with C^{14} amino acids by Drosophila supernatant fractions.

In vitro investigations of protein synthesizing systems in Drosophila have been carried out by Jenny, et al., (1962), and Fox, et al., (1965). The Tenebrio system has been extensively described and investigated by Ilan and Lipmann (1966), and Ilan (1968).

MATERIALS AND METHODS

I. Preparation of Drosophila Subcellular Fractions

The cell-free fractions of Drosophila were prepared by the method of Ilan (1968). Adult flies, which had been raised on corn meal-Karo-agar medium, were collected and stored at -50°C until used. One gm of flies was used per 3 ml of homogenizing medium, which contained: 0.035 M Tris-HCl, pH 7.6; 0.025 M KCl; 0.01 M $MgCl_2$; 0.007 M 2-mercaptoethanol; and 0.005 M phenylthiourea. Following homogenization in a Sorvall Omni-Mixer, the suspension was filtered through cheesecloth and centrifuged first at 20,000g for 20 minutes, then at 27,000g for 20 minutes. The supernatant fluid was then centrifuged at 105,000g for 2 hr. The post-microsomal supernatant fraction from this centrifugation was dialyzed overnight against 0.01 M Tris-HCl, pH 7.6, and 0.005 M 2-mercaptoethanol, frozen, and stored at -50°C until used as a source of supernatant factors. The microsomal pellets were gently homogenized by hand in 1% deoxycholate buffer containing 0.007 M 2-mercaptoethanol; 0.01 M $MgCl_2$; 0.035 M Tris-HCl, pH 7.6; and 0.5 M NH_4Cl, layered on a buffer containing 1 M sucrose; 0.5 M NH_4Cl; 0.035 M Tris-HCl, pH 7.6; 0.01 M $MgCl_2$; and 0.007 M 2-mercaptoethanol, and centrifuged at 105,000g for 2 hr. Ribosomes were washed with the above buffer, omitting the deoxycholate; the ribosomal pellets were frozen at -50°C until used.

Transfer RNA (tRNA) was prepared from Drosophila by phenol treatment (Gierer and Schramm, 1956) of the 105,000g supernatant fluid. The aqueous layer was separated by centrifugation and extracted twice with ether. Potassium acetate was added until its concentration in the solution was 2%. The tRNA was precipitated by the addition of 2 volumes of cold ethanol.

The tRNA was stripped of amino acids by incubation in 0.5 M Tris-HCl, pH 10.0 for 60 minutes at 35°C. The resulting reprecipitated tRNA was dissolved in 0.05 M phosphate buffer, pH 6.7 and dialyzed overnight against the same buffer. The tRNA was precipitated with 2 volumes of cold ethanol, dried, and stored at 4°C *in vacuo*, over KOH. Stripped calf liver tRNA was purchased from General Biochemicals, Chagrin Falls, Ohio.

Ribosomal RNA concentrations were measured by the methods of Warburg and Christian (1941); tRNA concentrations were measured spectrophotometrically, assuming 24 optical density units at A_{260} per mg RNA. Protein concentrations were determined by the method of Lowry, *et al.*, (1951).

II. C^{14} Amino Acid Incorporating System

The cell-free incorporating system is essentially that of Ilan and Lipmann (1966). In a total volume of 0.20 ml, the reaction mixture contained: 8 mM dithiothreitol; 30 mM Tris-HCl, pH 7.6; 6 mM $MgCl_2$; 0.5 mM GTP; 2.0 mM ATP; 8.0 mM phosphoenolpyruvate; 60 µg pyruvic kinase; 46 mM KCl; 19 C^{12} amino acids, 0.06 mM each; 0.2 µC C^{14} amino acid; 2 mg/ml ribosomal RNA (rRNA) of Drosophila ribosomes; and 0.2 mg supernatant protein. Incubation was carried out at 30°C for varying lengths of time, following which the reaction was stopped by the addition of 5 ml of 5% trichloroacetic acid (TCA). This was heated to 90°C for 20 minutes, then filtered through Millipore filters which have an average pore diameter of 0.45 µ. The filters were washed with 40 ml of 5% TCA, dried, and counted in a liquid scintillation spectrophotometer.

III. Aminoacylation of tRNA

The *in vitro* tRNA charging reaction mixture contained, in total volume of 0.10 ml: 30 mM imidazole, pH 7.0; 10 mM $MgCl_2$; 2.4 mM dithiothreitol; 2.4 mM ATP; 0.2 mg tRNA; 0.1 µC C^{14} amino acid; and 0.1 mg supernatant protein. Incubations were carried out at 30°C for 30 minutes, then stopped by the addition of 5 ml of cold 5% TCA. The mixture was then

filtered through Millipore filters, which were washed with 40 ml of cold 5% TCA, dried, and counted as above.

RESULTS AND DISCUSSION

A typical time curve for the incorporation of C^{14} leucine into hot TCA precipitable material by the Oregon-R (Ore-R) wild type system is shown in Figure 1. Incorporation is dependent upon the addition of the supernatant fluid, as shown by the difference between the complete system and the ribosomal fraction alone without the addition of supernatant fluid. Supernatant fraction alone has no activity. The rate of incorporation

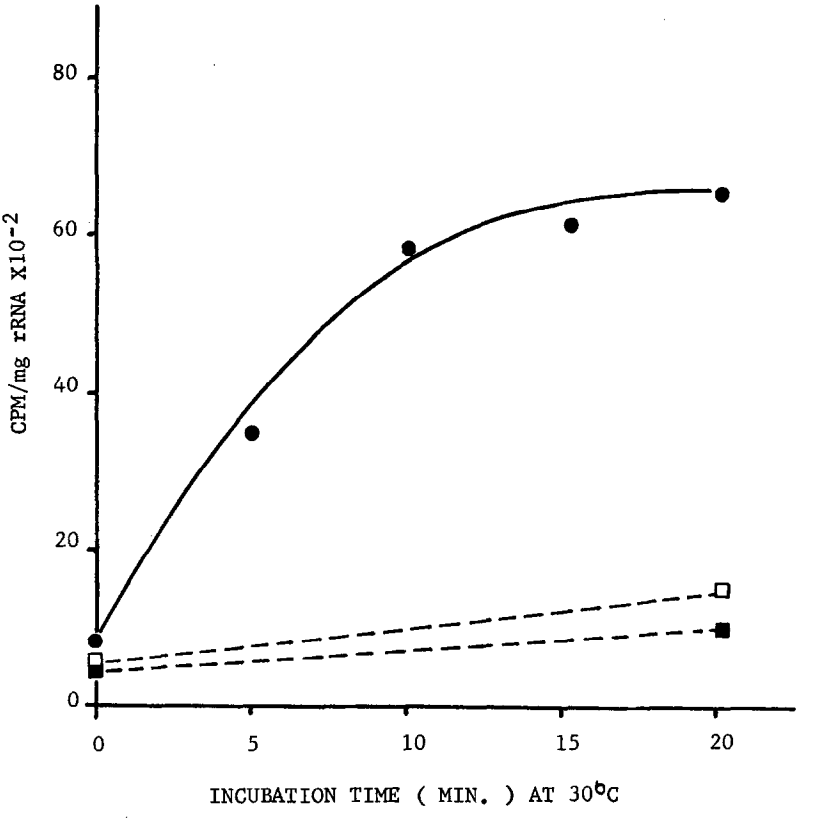

Fig. 1. Time curve for the incorporation of C^{14} leucine into hot trichloroacetic acid (TCA) precipitable material by Drosophila (Oregon-R) cell-free system as described in Methods. Complete system (●); ribosomes minus supernatant (□); supernatant without ribosomes (■).

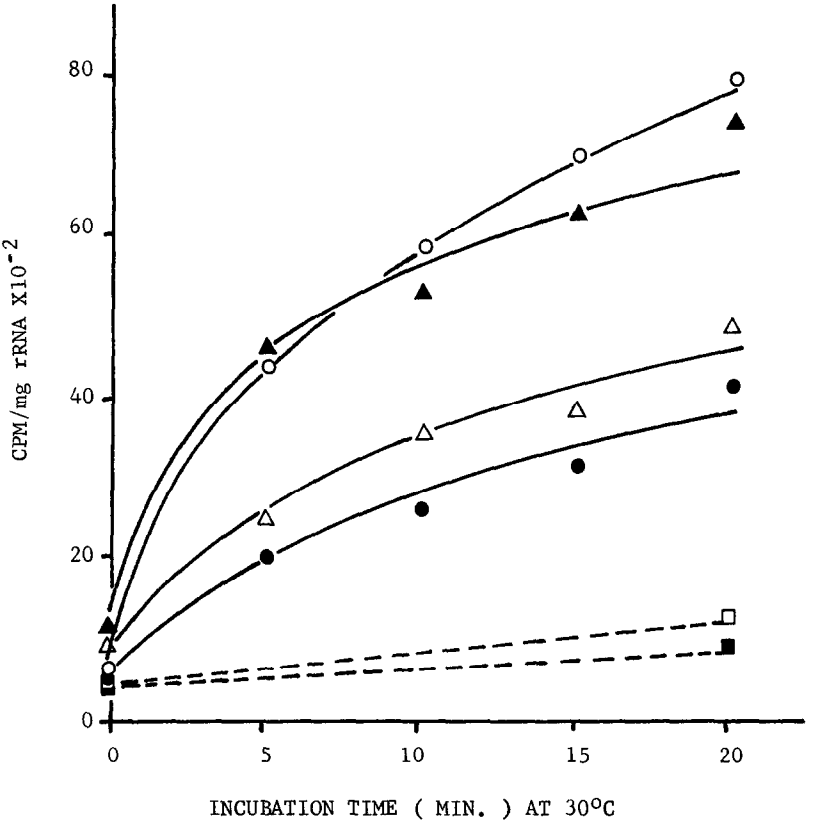

Fig. 2. Comparison of time curves of incorporation of C^{14} leucine into hot TCA precipitable material by cell-free systems of Oregon-R and A^{53g} Drosophila. Oregon-R ribosomes plus Oregon-R supernatant (●); Oregon-R ribosomes plus A^{53g} supernatant (○); A^{53g} ribosomes plus Oregon-R supernatant (△); A^{53g} ribosomes plus A^{53g} supernatant (▲). Oregon-R ribosomes only (□); and A^{53g} ribosomes only (■).

by the complete system is linear for 5 minutes and then proceeds at a decreasing rate.

Comparison of incorporation by extracts prepared from Ore-R wild type and $\underline{A^{53g}}$ flies is depicted in Figure 2. A marked difference in incorporation is noted with regard to the source of the supernatant fractions Supernatant fluid from the mutant preparation stimulates C^{14} leucine incorporation into hot TCA precipitable material regardless of the source of ribosomes.

TABLE 1

STIMULATION OF C^{14} AMINO ACID INCORPORATION BY $\underline{A^{53g}}$ SUPERNATANT FRACTION

	Incorporation			
	Supernatant Fraction			
	A^{53g}		Ore-R	
	Ribosomes			
	A^{53g}	Ore-R	A^{53g}	Ore-R
	CPM/mg rRNA			
Phenylalanine	5,723	6,120	3,331	3,343
Leucine	7,389	11,366	3,394	3,161
Proline	4,722	4,508	2,530	2,958
Glutamic Acid	14,767	17,596	9,275	11,042
Valine	5,923	7,719	2,319	2,462

Incubation at 30^0C for 15 minutes as described in Methods. Supernatant fractions from Ore-R or $\underline{A^{53g}}$, 0.2 mg protein, were added to each tube which contained either Ore-R or $\underline{A^{53g}}$ ribosomes, 2 mg rRNA/ml, as indicated, and 0.2 μC of the C^{14} amino acid to be tested.

The results of incorporation studies using five different C^{14} amino acids by this system are summarized in Table 1. Stimulation of incorporation by the mutant supernatant fraction can be seen for all five C^{14} amino acids tested.

Since the stimulatory effect of the mutant system is confined to the supernatant fraction, rather than the ribosomes, the $\underline{A^{53g}}$ and Ore-R supernatant fluids were tested with regard to their ability to charge tRNA with C^{14} leucine, as described in Methods. Table 2 shows that the $\underline{A^{53g}}$ supernatant fraction charges both calf liver tRNA and Drosophila tRNA to a greater extent than does the Ore-R wild type supernatant fluid.

Comparative studies of C^{14} amino acid incorporation into hot TCA

TABLE 2

STIMULATION OF CHARGING OF DROSOPHILA AND CALF LIVER tRNA WITH C^{14} LEUCINE BY A^{53g} SUPERNATANT FRACTION

	Incorporation	
	Supernatant Fraction	
	Ore-R	A^{53g}
	CPM/mg tRNA	
Drosophila tRNA	5,031 (120)	10,138 (129)
Calf Liver tRNA	3,833 (110)	8,746 (109)

Incubation conditions as described in Methods. Supernatant protein, 0.1 mg, from Ore-R or A^{53g} was added to each tube as indicated. Drosophila or calf liver tRNA, 0.2 mg per tube, was used as shown. Numbers in parenthesis represent controls to which no tRNA was added and are expressed as CPM per incubation tube.

precipitable material by extracts of Ore-R and A^{53g} flies have shown that there is a difference in incorporation, and that the source of this difference is confined to the post-microsomal supernatant fluid. These results indicate that the A^{53g} mutant affects protein synthesis at the translational level and that this effect is involved with the ability of the constituents of the post-microsomal supernatant fluid to charge tRNA with amino acids. Work is continuing to further define this effect.

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