In vitro suppression of a nonsense mutant of *Drosophila melanogaster*

Eric Kubli*, Thomas Schmidt*, Presley F. Martin[†] and William Sofer[†]

*Zoologisches Institut der Universität Zürich-Irchel, Winterthurerstrasse 190, CH-8057, Zürich, Switzerland, and [†]Waksman Institute of Microbiology, Rutgers University, Piscataway, NJ 08854, USA

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ABSTRACT

When RNA isolated from the *Drosophila melanogaster* alcohol dehydrogenase (ADH) negative mutant CyOnB was translated "in vitro" in the presence of yeast opal suppressor tRNA, a wild type size ADH protein was obtained in addition to the mutant gene product. This identifies the CyOnB mutant as an opal (UGA) nonsense mutant. From the molecular weight of the mutant protein, and from the known sequence of the ADH gene (Benyajati et al., Proc.Natl.Acad.Sci. USA 78,2717-2721,1981), we conclude that the tryptophan codon UGG in position 234 has been changed into a UGA nonsense codon in the CyOnB mutant. Furthermore, we show that the UAA stop codon of the wild type ADH gene is resistant to suppression by a yeast ochre suppressor tRNA. This is in contrast to the high efficiency of suppression of the CyOnB UGA nonsense codon, despite an almost identical codon context.

INTRODUCTION

Nonsense mutants and their suppressors have played a major role in the elucidation of gene function in bacteria and yeast^{1,2}. Despite great efforts, progress in the identification of this type of mutant in higher eukaryotes has been rather slow. Nonsense mutants have been described in cultured mammalian cells^{3,4}, their viruses^{5,6} and most recently in the β -globin gene of humans^{7,8}. Moreover, excellent genetic evidence has been presented for the existence of a nonsense mutant⁹, and informational suppressors^{9,10} in the nematode Caenorhabditis elegans.

Drosophila melanogaster is one of the organisms of choice among higher eukaryotes for the screening of nonsense mutants and tRNA nonsense suppressors for two major reasons. First, chemical selection schemes have been developed for several enzymes, which enormously facilitate the screening of forward and backward mutations¹¹⁻¹⁴. Second, the ability to localize

the genes coding for tRNAs by "in situ" hybridization of labeled tRNAs to polytene salivary gland chromosomes narrows down considerably the class of suppressor mutants which might act as tRNA dependent nonsense suppressors¹⁵. In this paper we describe the identification of the first nonsense mutant in Drosophila melanogaster.

MATERIALS AND METHODS

RNA was isolated from 1 g each of CyO/Df(2L)64j (wild type ADH) and CyO^{nB}/Df(2L)64j (mutant ADH) flies by the hot phenol extraction method¹⁶. High molecular weight RNA was precipitated with 3 M LiCl¹⁶ and used as a translation probe. A standard reticulocyte incubation mixture was used for translation with a total volume of 30 μ l¹⁶. [³H]-leucine or [³H]-lysine were used as labeled amino acids as described in Figs. 1 and 2. The [³H]-leucine concentration was 0.18 nmol (NET 460, 110 Ci/mmol, New England Nuclear), the [³H]-lysine concentration 0.23 nmol (TRK 520, 87.3 Ci/mmol, Radiochemical Centre Amersham). After 90 min incubation at 30° C 2 μ l were used for measurement of the incorporation of the labeled amino acid into protein¹⁷, 5 μ l were used directly for analysis on a 12.5 % PAA-gel¹⁸ (after addition of 2 μ l β -mercaptoethanol, 2 μ l of 20 mM l-leucine (or l-lysine), 5 min boiling). The remaining 23 μ l was used for antibody precipitation by adding 25 μ l of NET buffer (10 mM Tris-HCl pH 7.5; 100 mM NaCl; 1 mM EDTA; 20 mM l-leucine or l-lysine), transferring the probes into siliconized Eppendorf tubes, adding 5 μ l goat anti-ADH serum¹⁹ and incubating at room temperature for 3 h. Fifty μ l of a 10% suspension of formaldehyde fixed Staphylococcus aureus in TENT buffer (25 mM Tris-HCl, pH 7.5; 10 mM EDTA pH 7.5; 0.35 M NaCl; 1% Triton X-100) were added, vortexed thoroughly and the mixture incubated on ice for 15 min. The Staphylococcus-antibody-antigene complex was pelleted by centrifugation, the pellet resuspended in 1 ml TENT buffer and transferred into new, siliconized Eppendorf tubes. This procedure was repeated 4 times at 0° C. After the final centrifugation 20 μ l sample buffer²⁰, 5 μ l β -mercaptoethanol, and 5 μ l 20 mM l-leucine (or l-lysine) were added, boiled for 5 min and after centrifugation the supernatant loaded on a 12.5% SDS PAA-gel¹⁸

and fluorographed²¹ for 4 d (Fig. 1) or 17 d (Fig. 2). The yeast wild-type and suppressor tRNAs were purified as described previously^{22,20}.

RESULTS AND DISCUSSION

Although many null activity mutants have been described in Drosophila melanogaster²³, none, so far, have been identified as nonsense mutants. Considering the fact that the alcohol dehydrogenase system allows a sensitive and efficient screening both for enzyme negative and positive mutants^{24,25} we have chosen one of the ADH null mutants for a detailed study (ADH: alcohol:NAD⁺ oxidoreductase, EC 1.1.1.1; ADH refers to the enzyme, Adh to the ADH gene). CyO^{nB} has been described as an EMS induced²⁶, enzyme-negative, cross reacting material (CRM) positive ADH mutant²⁷. The presence of CRM and its slightly lower molecular weight compared to ADH isolated from wild-type flies (Fig. 1b) suggest, that the putative nonsense mutation is localized in the C-terminal part of the protein. Starting from the fact that yeast nonsense suppressor tRNAs can suppress eukaryotic nonsense codons "in vitro"²⁸ and "in vivo"^{20,29}, we reasoned that it might be possible to readthrough the putative nonsense codon in the CyO^{nB} ADH mRNA by its cognate suppressor tRNA. Translation of RNA isolated from CyO (the ADH positive strain from which CyO^{nB} was derived) in a reticulocyte cell free protein synthesizing system, followed by anti-ADH antibody precipitation and SDS-polyacrylamide gel electrophoresis, yields a 26 KD protein (Figs. 1a and b, lanes c). The size of this protein corresponds to the molecular weight predicted from both the known sequence of the ADH gene³⁰ as well as the amino acid sequence of the ADH protein³¹. RNA isolated from CyO^{nB} flies induces the synthesis of a slightly shorter protein of approximate 24 KD molecular mass (Figs. 1a and b, lanes d). Neither amber nor ochre yeast suppressor tRNA addition influenced the size of the protein programmed by this RNA preparation (Figs. 1b, lanes e-h). However, incubation of RNA isolated from CyO^{nB} together with opal suppressor tRNA clearly produces two bands (Figs. 1a and b, lanes i-m). One band corresponds to the mutant protein, the other to wild-type ADH size. This result identifies the ADH CyO^{nB} mutant as an UGA nonsense

Figure 1 Identification of CyO^{NB} as an Adh opal nonsense mutant. analysis of "in vitro" translation products on a 12.5% PAA-gel with subsequent fluorography. a) Total protein synthesized in the reticulocyte cell free system. b) Same, but after anti-ADH antibody precipitation. Lanes a+n: MW markers (* = carbonic anhydrase, 30 KD): b: control, no exogenous RNA added; c: with CyO RNA (25 μ g, wild-type); d: with $CyONB$ RNA (16 μ g, mutant); e+f: with $CyONB$ RNA and yeast amber suppressor tRNA-Tyr (1 μ g/2 μ g); g+h: $CyONB$ RNA and yeast ochre suppressor tRNA-Tyr (1 μ g/2 μ g); i-m: $CyONB$ RNA with yeast opal suppressor tRNA-Ser (240 ng/720 ng/1.2 μ g/1.8 μ g). [3H]-leucine was used as a labeled amino acid. Pointers indicate position of wild-type ADH (26 KD) and mutant ADH (24 KD). Addition of opal suppressor tRNA results in the synthesis of a readthrough protein of wild-type size, thus identifying $CyONB$ as an opal (UGA) nonsense mutant.

mutant. From the molecular mass of the mutant protein (24 KD), the number of missing peptides (Martin et al., in prep.), and the fact that the transition inducing mutagen EMS³² had been used for mutagenesis³⁰ it is likely that the tryptophan codon in position 234 has been changed into a UGA nonsense codon in the CyO^{nB} mutant. All other mutants leading to a protein of similar size via nonsense codons demand either a transversion or the exchange of two base pairs.

The high efficiency of suppression of the UGA nonsense codon by the yeast opal suppressor tRNA is quite remarkable (Fig. 1b, lanes i-m). A comparison of the opal nonsense mutant codon context with the context of the wild typ termination signal (UAA) shows that 5 out of 6 nucleotides are identical (Table 1). Therefore, we wondered whether the Adh wild-type stop codon could be suppressed with equal efficiency by an ochre suppressor tRNA. Readthrough of the UAA stop signal should lead to the synthesis of a 31 KD protein³⁰. [³H]-lysine was chosen as a labeled amino acid in order to increase the specific activity of the readthrough protein (5 lysine residues are encoded by the wild-type Adh gene, 6 additional lysines are encoded by readthrough of the UAA stop codon¹⁹). No readthrough could be detected with ochre suppressor tRNA (Fig. 2b, lanes e, m, and n). The absence of readthrough cannot be due to a failure to precipitate the readthrough product by the antibody because no change in the pattern of the total protein synthesized was observed in the 31 KD region (Fig. 2a, lanes e, m, and n). Neither can it be due to a nonfunctional ochre suppressor tRNA, since two different experiments have de-

Table 1 Codon context sequences.

Generalized eukaryotic UGA ₃₃ termination codon context	NAN UGA Pu Py N
<u>CyO^{nB}</u> UGA context*	AUC UGA AAA
Generalized eukaryotic UAA ₃₃ termination codon context	$\frac{G}{C}NN$ UAA Pu Py N
Adh stop codon context ¹⁹	AUC UAA GAA
* this paper and Benyajati and coworkers ¹⁹ .

Figure 2 Readthrough of the Adh UAA stop codon. Analysis of "in vitro" translation products on a 12.5% PAA-gel with subsequent fluorography.
a) Total protein synthesized in the reticulocyte cell free system.
b) Same, but after anti-ADH antibody precipitation.
Lanes a+o: MW markers (* = carbonic anhydrase, 30 kD); lanes b+f: controls, no exogenous RNA added; lanes c+g: with *CyO* RNA (23 µg); lanes d, k+l: *CyO* RNA with yeast amber suppressor tRNA-Tyr (3 µg/1 µg/3 µg); lanes e, m+n: *CyO* RNA with yeast ochre suppressor tRNA-Tyr (3 µg/1 µg/3 µg); lanes h+i: *CyO* RNA with yeast tRNA-Tyr wild-type (1 µg/3 µg). Pointers indicate position of wild-type ADH. [³H]-leucine was used as a labeled amino acid in lanes b-e, [³H]-lysine for the assays of lanes f-n. No readthrough protein is observable in the 31 kD region (predicted from the ADH gene sequence³⁰) indicating that the UAA stop codon cannot be suppressed with equal efficiency as the UGA nonsense codon, despite an almost identical codon context.

monstrated the activity of this probe. First, the UAA stop codon of the Q_{β} replicase³³ could be suppressed "in vitro" (unpublished). Second, it has been shown that upon injection of this probe into Xenopus laevis oocytes suppressor specific changes in the protein pattern can be induced²⁹. The obvious conclusion is that the UAA stop codon does not allow a readthrough with the same efficiency as the UGA nonsense codon, despite an almost identical codon context. It has been shown that eukaryotic termination signals are difficult to suppress "in vivo"²⁹. One factor responsible for this phenomenon might be the stop codon context³³. Comparison of the context surrounding the UGA nonsense codon with the typical eukaryotic context³³ shows that the statistically highly significant A in the second position before the termination signal is replaced by an U in the mutant (Table 1). Furthermore, it has been suggested that an adenine residue adjacent to the 3' side of UGA signals inefficient termination of polypeptide chain synthesis in prokaryotes³⁴. This violation of the UGA context rules by the CyO^{NB} mutant might explain the above mentioned observation of about 50-60% readthrough by the opal suppressor tRNA. The UAA context seems to be less prone to suppression due to a more flexible "correct" codon context creating a strong termination signal. Thus the differential efficiency of suppression of the two stop codons can be interpreted in terms of specific codon contexts needed for an efficient termination of polypeptide chain synthesis.

The following two facts seem to indicate that it might be rewarding to use this mutant for screening for opal nonsense suppressors in Drosophila. First, the UGA triplet seems to be easily suppressed and, due to the high amount of ADH mRNA (1-3% in the poly A⁺ RNA¹⁹), the ADH synthesized easily observed. Second, the sensitive ADH selection system allows the screening of a large number of mutant gametes, with a relative low level of enzymatic activity. The identification of this nonsense mutant could lead to a successful search for opal suppressor tRNAs in Drosophila melanogaster, thus providing the tools which have been of great importance in the study of gene expression in prokaryotes and yeast.

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