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A mutation at the start codon defines the differential requirement of *dpy-11* in *Caenorhabditis elegans* body hypodermis and male tail

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Abstract

dpy-11 encodes a thioredoxin-like molecule that is important for both body and male sensory ray morphogenesis in *Caenorhabditis elegans*. A mutant allele, *s287*, has a point mutation with its start codon, AUG, converted into AUA, presumably leading to null function. Since only a weak loss-of-function phenotype was observed, we tested whether an alternative start codon or the converted AUA could be used for translation initiation with reduced efficiency. Based on a functional assay of mutant phenotype complementation and biochemical analysis examining the in vivo synthesis of wild-type and mutant proteins, we conclude that AUA can be used as a less-efficient start codon for initiating translation of DPY-11. Our results also provide direct evidence that the body hypodermis and male tail of *C. elegans* have differential requirements of *dpy-11* activity for their respective normal morphogenesis.
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The translation of eukaryotic mRNA in the cytoplasm begins after a start codon is selected by the translation machinery at the initiation phase. Methionine encoding AUG is usually used as the translation start codon because of the stable interaction between the AUG codon on mRNA and CAU anticodon on methionyl-tRNA [1]. In the selective recognition of this start codon in prokaryotes, the presence of a Shine–Dalgarno sequence in front of this AUG triplet dictates the entry of ribosomal subunits. In eukaryotes, the AUG start site relies heavily on the initiating methionyl-tRNA with a CAU anticodon [1]. Small 40S ribosomal subunits and various initiation factors (eIFs) cooperatively engage with the 5′ cap of mRNA and migrate directionally from the 5′ towards the 3′ end until the CAU anticodon of methionyl-tRNA first encounters the AUG start codon on the mRNA. This scanning process is followed by the joining of the 60S ribosomal subunit and the subsequent elongation event leading to the synthesis of a polypeptide product [2–4]. In all prokaryotic and eukaryotic systems, the AUG codon is usually used for translation initiation [5]. Nevertheless, there are some exceptions in mitochondria, plastids, viruses, bacteria, and some plants, where alternative codons can be used to initiate translation [6–13].

In our previous study, we showed that DPY-11 of *Caenorhabditis elegans* is a membrane bound thioredoxin-like enzyme that is important for both body and sensory organ morphogenesis [14]. This protein associates with the ER/Golgi network and could be involved in the modification of collagen molecules. Through the characterization of a number of mutant alleles, we showed that the membrane anchorage is of paramount importance for the proper function of DPY-11. The thioredoxin domain, putatively serving as a catalytic unit, is essential for *dpy-11* activity. We also revealed in some mutant alleles that the morphogenesis of male sensory organs and body shape can be dissociated, presumably due to a lower sensitivity of the male tail to the reduction of *dpy-11* activity. On the other hand, it is equally possible that the specific transmembrane domain of DPY-11 protein is not required for male sensory ray development.

In one of the mutant *dpy-11* alleles, *s287*, the AUG start codon is mutated to AUA and a weak loss-of-

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function phenotype, mild dumpy body, is displayed. The phenotype suggests that the activity of this mutant allele must not be completely eliminated. There may be different reasons for such hypomorphic features, including the mutant allele picking up an alternative start codon, promiscuous use of AUA as the start codon or transcription attenuation by the mutation. In this study, we provide evidence that this reduced *dpy-11* activity in *s287* mutation is due to the use of AUA as a substituting and inefficient start codon. The result of this characterization supports the notion that the AUG start codon is not obligatory for translation initiation, although it confers the optimal initiation efficiency. Our results also provide direct molecular and biochemical evidence that the threshold level of the *dpy-11* requirement is lower in male tail morphogenesis than in body shape differentiation.

Materials and methods

Worm cultures. Worm strains were cultured and maintained as described by Brenner [15] at 20 °C unless otherwise stated. All *dpy-11* alleles were either obtained from *Caenorhabditis* Genetics Center, Minnesota or provided by the Baillie laboratory.

Phenotypic analysis of dpy-11 alleles. All *dpy-11* mutants were characterized by their respective body and male tail phenotype under Nomarski microscopy. Except the reference allele *e224*, which was coupled with *him-5*(*e1490*)*V*, all alleles were in *him-8*(*e1489*)*IV* background [16]. The average body length was obtained by measuring 30 males with a micrometer. Their ray phenotypes were recorded using a Ram Index defined by the degree of sensory ray swelling, with 0 indicating wild-type and 5 representing the most severe one as described previously [14]. Thirty males of each allele were scored and the value was averaged.

Mapping the lesion in dpy-11(s287) mutant allele. The genomic DNA of the *dpy-11*(*s287*) allele was extracted as described by Sulston and Hodgkin [17]. About 400–600 ng of genomic DNA was used for PCR amplification with *dpy-11* gene specific primers. The PCR product was sequenced with the BigDye Terminal Sequencing Kit (Perkin–Elmer) using the same primer sets [14].

Engineering of expression and deletion constructs. The constructions of plasmids p*d11*ΔSP, p*d11*cDNA, and p*d11*AGFPC have been described previously [14]. The 5′ genomic fragment of the *s287* mutant was amplified by PCR with the KC211 and KC247 primers and cloned into a pSP72 vector at the *Eco*RI and *Eco*RV sites to generate p72ATA2. An *Acc*I fragment of p72ATA2 was subcloned into p*d11*cDNA at the *Acc*I site and an extra *Ppu*MI fragment was deleted by self-ligation as p$d11^{ata}$cDNA. The promoter of p*d11*AGFPC was swapped with the *Sac*I fragment from p$d11^{ata}$cDNA to generate p$d11^{ata}$AGFPC. All these constructs were transformed into the null allele, *e207*, and the reference allele, *e224*, with pRF4 or p*d13* GFP markers. The rescue activity of these constructs and their signals were examined in at least three different transgenic lines.

RT-PCR. One plate of each strain of worms (~500 worms) was collected and stored at −80 °C before the total RNA was extracted. The total RNA was extracted with TRIzol reagent (Invitrogen, cat. no. 15596). The amount of total RNA was quantified by spectrophotometer at the wavelength of 260 nm. Five microgram of total RNA was used for first strand synthesis with Superscript II reverse transcriptase (Invitrogen, cat. no. 18064–022) followed by RNaseH treatment. To reveal the expression of transgenes, five microlitres of each first-strand product was used for PCR with *gfp* specific primer KC142 (5′-CCAGTGAAAAGTTCTTCTCC-3′) and *dpy-11* specific primer KC214 (5′-CCAGACTACAAGCATCC-3′) in an annealing temperature of 54 °C for 30 cycles. The expression of an unrelated collagen gene *dpy-13* in both strains served as an internal reference control and was also monitored by RT-PCR with gene-specific primers KC254 (5′-CGGCACGAGCCATGGACATTG-3′) and KC255 (5′-GCGTC AGATCCTGGC-3′). These PCR products were quantified in 1% agarose gel.

Protein extraction and Western blot analysis. Five to 10 plates of each strain of transgenic worms (~2500–5000 worms) were collected and stored at −80 °C before protein extraction. The packed worms were homogenized with 300 μl lysis buffer (1 mM DTT, 1 mM EDTA, 50 mM NaCl, 1% NP40, 1 mM PMSF, 2% SDS, and 20 mM Tris, pH 8.0) and boiled at 100 °C for 5 min. The supernatant was collected. The amount of total protein was quantified by a Bio-Rad DC Protein Assay (cat. no. 500–0116). Sixty-five micrograms of total protein was used for a 7.5% SDS–PAGE analysis. The separated samples were transferred onto a nitrocellulose membrane (Bio-Rad. cat. no. 170–3930) for Western blot analysis. Blocking was performed with 5% non-fat milk powder in PBST for 2–3 h, followed by further incubation in 10 ml of a blocking solution with anti-GFP antibody (0.4 μg/ml, Roche, cat. no. 1814460) overnight. The membrane was washed twice with PBST and incubated in goat anti-mouse antibody conjugated with alkaline phosphatase (4:10,000) for 1 h. The signal was detected by a Bio-Rad Immuno Blot Assay (cat. no. 170–6461).

Results

Phenotypic characterization and mutation mapping of the s287 allele

Mutations of the *dpy-11* gene in *C. elegans* led to a dumpy body morphology as well as a specific male sensory ray defect, which has often been referred to as lumpy ray (Fig. 3). Correlation of the phenotypes with specific mutant alleles of *dpy-11* suggests that these mutant phenotypes are dependent on the gene dosage. Maintenance of the wild-type body shape requires a higher level of *dpy-11* activity [14]. The null allele, *e207*, with the fourth codon changed to the *opal* stop codon, results in the most extreme mutant phenotype, i.e., severe body dumpiness and very swollen sensory rays (Table 1). In loss-of-function mutations, e.g., the *e224* allele with a point mutation converting glycine to glutamic acid at residue 76 within the thioredoxin enzymatic domain, the body dumpiness is much milder, so is the lumpiness of the male sensory rays. One *dpy-11* mutant allele, *s287*, was generated by ethylmethanesulfonate (EMS) under the background of reciprocal translocation of linkage groups *III* and *V*, *eT1*(III;V) [18]. Mapping of the lesion in the *s287* allele genomic DNA revealed that the AUG start codon was mutated to AUA (Fig. 1). No other mutation was detected in the entire coding region and one kilobase upstream of the authentic transcription start site. Interestingly, this mutant allele displayed tapered wild-type sensory rays while the body showed mild dumpiness (Figs. 3B and B′). This less-severe body abnormality and the normal rays indicate that this *s287* mutation is not a null

Table 1
Phenotypes of *dpy-11* mutant alleles and transgenic animals

	Allele[a]	Transgene	Mutation (amino acid changes)	Male Tail Ram index (0–5)	Male body length (mm)
1	Wild-type	—	—	0.0	0.88 ± 0.04
2	*s287*[b]	—	M1I	0.0	0.63 ± 0.04
3	*e395*[b]	—	G187E	0.0	0.65 ± 0.04
4	*e431*[b]	—	G187E	0.1	0.65 ± 0.03
5	*e224*[b]	—	G76E	1.9	0.63 ± 0.02
6	*e224*	p*d11*cDNA	G76E	0.0	0.87 ± 0.03
7	*e224*	p*d11*AGFPC	G76E	0.0	0.82 ± 0.06
8	*e224*	p*d11*ΔSP	G76E	2.6	0.58 ± 0.06
9	*e224*	p*d11*^{ata}cDNA	G76E	1.2	0.63 ± 0.05
10	*e224*	p*d11*^{ata}AGFPC	G76E	1.2	0.58 ± 0.05
11	*e207*[b]	—	R4*Opal*	4.5	0.46 ± 0.03
12	*e207*	p*d11*cDNA	R4*Opal*	0.0	0.80 ± 0.06
13	*e207*	p*d11*AGFPC	R4*Opal*	0.0	0.74 ± 0.07
14	*e207*	p*d11*ΔSP	R4*Opal*	4.4	0.45 ± 0.04
15	*e207*	p*d11*^{ata}cDNA	R4*Opal*	1.2	0.56 ± 0.06
16	*e207*	p*d11*^{ata}AGFPC	R4*Opal*	1.2	0.53 ± 0.06

[a] In this study, all strains have *him-8*(*1489*) mutation to increase the male animals in the population by sex chromosome non-disjunction [16].
[b] The phenotypes of these strains were reported previously [14].

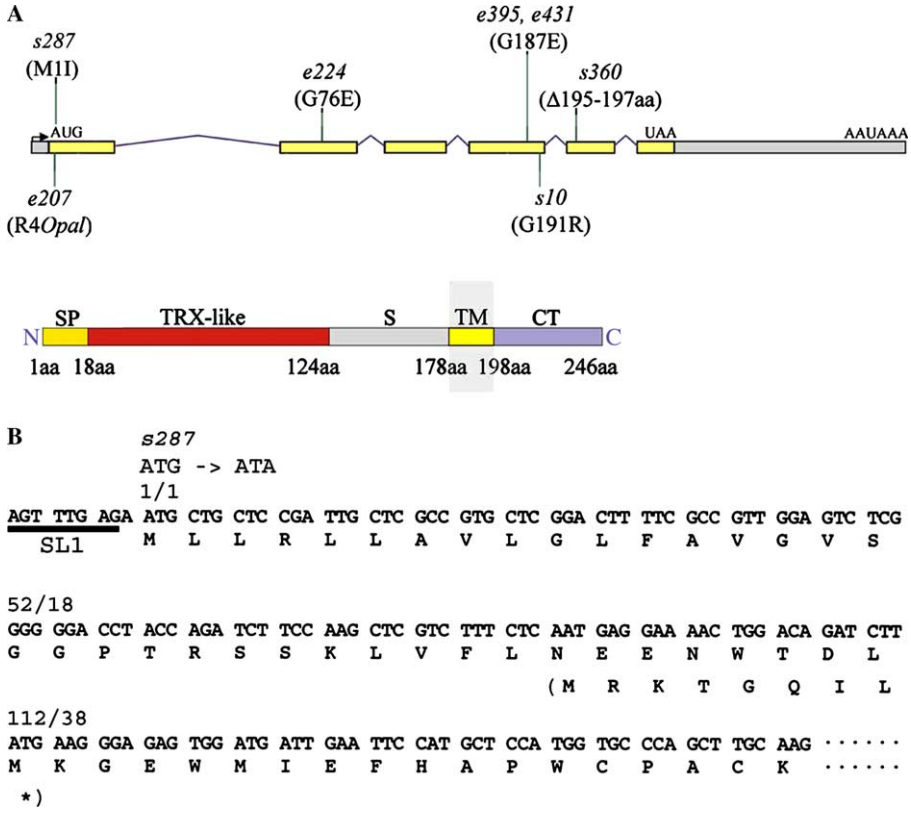

Fig. 1. (A) Schematic diagram of *dpy-11* showing the mapped lesions in different alleles (upper) and the domain structure of its encoded product (lower). (B) 5′ sequence of the *dpy-11* transcript with the *s287* mutation and the encoded amino acid sequences depicted underneath the nucleotide sequence. The out-of-frame translated product is marked in brackets.

mutation. Since animals with some other mild loss-of-function alleles, e.g., *e395* and *e431*, present similar phenotypes, it is apparent that residual gene activity is present. There could be three possible explanations to account for this *s287* phenotype. When the AUG codon was mutated to AUA, an alternative AUG could have been used as the translation start. However, as an SL1 sequence is found trans-spliced immediately in front of

the start codon, no alternative AUG upstream of the authentic start is available (Fig. 1B). This specific explanation is unlikely. An out-of-frame AUG of about 60 nucleotides 3′ to the authentic start codon may be used. Yet, the so-synthesized protein would be a short polypeptide irrelevant to *dpy-11* function. On the other hand, if a further downstream AUG corresponding to the methionine 38 of DPY-11 was used, a truncated protein lacking the signal peptide but with a complete functional thioredoxin domain would be made. This truncated protein may still be able to orient its transmembrane domain based on the "positive inside" rule [19]. Thus, a subpopulation of the truncated products may be displayed in the right topology and result in a low level of *dpy-11* biological activity. Another possibility is that the mutated AUA could still be used as a translation start and be recognized at an attenuated efficiency by the translation initiation factor complex and the anti-sense CAU codon on methionyl-tRNA. Hence, a reduced level of the functional product is made while the generated protein product retains its wild-type biological function. The third possibility, which cannot be ruled out at the moment, is that the G to A alteration at the *dpy-11* locus reduces *dpy-11* activity through attenuation of the transcription initiation or by reducing the mRNA stability.

DPY-11 is non-functional without a signal peptide

To assess these possibilities, a wild-type construct and two mutant constructs, p*d11*cDNA, p*d11*^{ata}cDNA, and p*d11*ΔSP, were engineered (Fig. 2). These transgene constructs were tested for their biological activity in a null mutant background to reveal their functional properties. When mutant construct p*d11*^{ata}cDNA with the start codon mutated to AUA was introduced into the *e207* animals, the body of the transformant animals was restored to medium dumpiness (0.56 mm; Fig. 2 and Table 1, line 15). Ten percent of the animals had wild-type sensory rays and gave an average Ram Index (R.I.) of 1.2 ($N = 30$). This result contrasts with that of the null (*e207*) animals, which show severe dumpiness (0.46 mm) and swollen rays (R.I. of 4.5, $N = 30$). When the

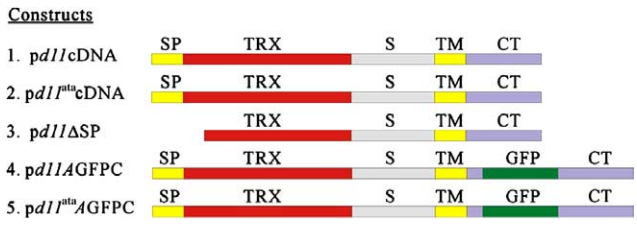

Fig. 2. Schematic diagram of constructs used in this study for analyzing the *dpy-11* expression. ATG and ATA represent the start codons used for the constructs and the green line represents the *gfp* gene insertion for visualization of the expression level. The *gfp* reporter is not drawn to the same scale.

p*d11*^{ata}cDNA was used to transform the reference allele, *e224*, the transgenic animals also showed similar rescued phenotypes at a comparable rate (Table 1). These results suggest that the transgene construct carrying the AUA start codon can code for a protein with the biological activity sufficient for complete restoration of wild-type male tail phenotype. The activity, however, is not sufficient to generate an animal with a normal body shape.

In order to test our first hypothesis of an alternative usage of the start codon, the possible downstream AUG start codon of the transcript was analyzed for its function (Fig. 1B). The out-of-frame AUG codon downstream of the authentic start was not tested. A further downstream AUG codon (M38) is in the same reading frame as the DPY-11 protein (ORF). Even though the signal peptide is lost, the catalytic thioredoxin domain is intact and may confer residual activity should it be displayed in the correct topology in the cell. To test for any residual function of this truncated protein, a p*d11*ΔSP construct was generated so that the region between the two in-frame AUG codons was deleted. This transgene utilizes the authentic translation initiation start and synthesizes a truncated protein without the signal peptide. This product counts on the "positive inside rule" for proper orientation. When this construct was introduced into both mutant *e207* and *e224* alleles, absolutely no rescue activity was detected (Table 1, lines 8 and 14). Therefore, a DPY-11 molecule lacking the signal peptide apparently failed to orient itself properly and did not behave as a functional protein. The results argue against the first hypothesis of using an alternative AUG as the start codon and cannot account for the *s287* mutant phenotype.

Mutant DPY-11(AUA) protein is synthesized at a reduced level

As the construct p*d11*^{ata}cDNA could provide rescue activity while signal peptide deletion construct, p*d11*ΔSP, could not, we suggested that the AUA codon might still serve as a less-efficient start codon in translation. To prove this hypothesis, we need to show that the protein of the correct molecular weight is produced. *gfp* tagged *dpy-11* fusion construct, p*d11*AGFPC, and the mutant version, p*d11*^{ata}AGFPC, were made and transformed into wild-type animals and *dpy-11* mutant animals to address this issue. It is obvious that both of the fusion constructs were functional. The null mutant rescue activities of these two *gfp* tagged constructs were similar to that of their non-*gfp*-tagged genes (Table 1, lines 6 and 7, 9 and 10, 12 and 13, 15 and 16). Wild-type *dpy-11* constructs with or without *gfp* tag showed 67.4% and 81.4% rescue efficiency, respectively, in null mutant animals when body morphology was used as the assessment criterion. Both transgene versions completely restored the wild-type sensory ray features (Table 1,

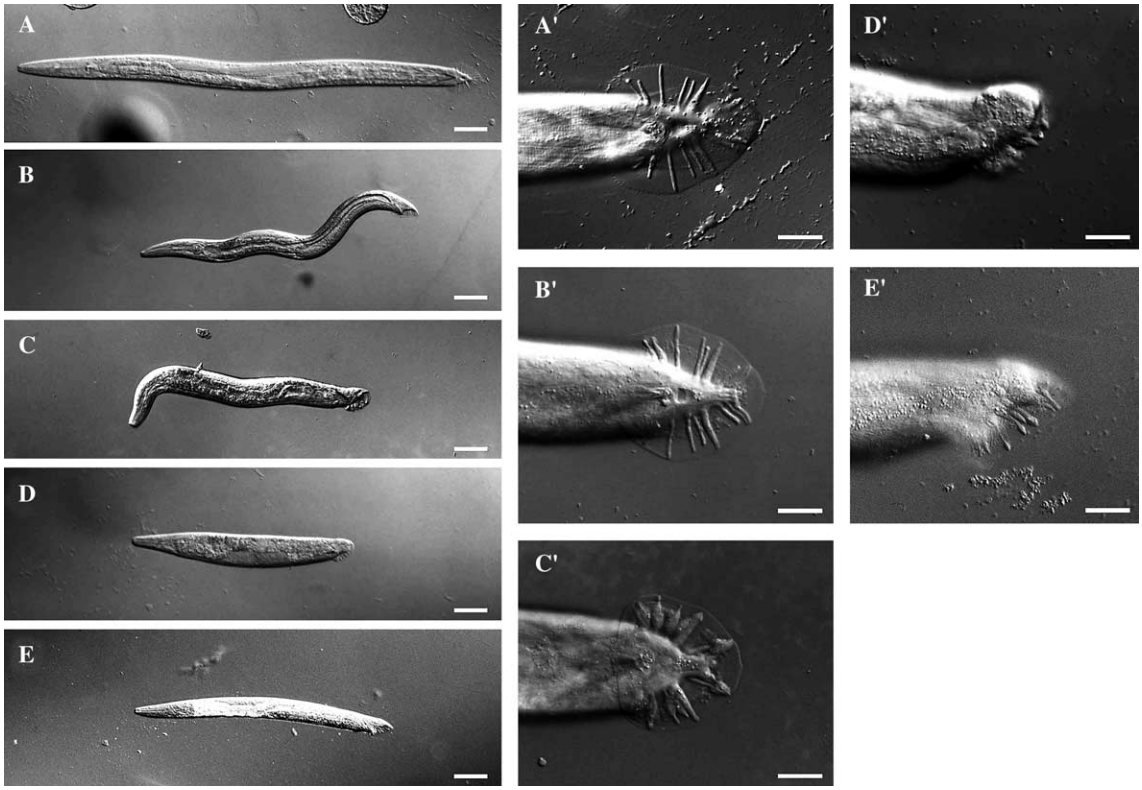

Fig. 3. Phenotypic characterization of *dpy-11* mutants and transgenic animals. A and A′, B and B′, C and C′, D and D′ came from wild-type, *s287*, *e224*, *e207* mutant animals, respectively. E and E′ depict transgenic animals with the transgene p*d11*^{ata}AGFPC. The scale bar for A to E = 50 μm and A′ to E′ = 20 μm.

lines 12 and 13, also lines 6 and 7). On the other hand, the p*d11*^{ata}AGFPC and p*d11*^{ata}cDNA transgenes showed body morphology rescue efficiency at 18.6% and 25.6%, respectively (Table 1, lines 15 and 16), displaying only one-third of the efficiency of their wild-type counterparts. Both of them could not confer complete rescue of the sensory ray abnormality (Table 1, lines 15 and 16).

When the expression level and profiles of these *gfp*-tagged DPY-11 proteins were examined with fluorescent microscopy [14], the signal of the wild-type fusion, p*d11*AGFPC, was strong in the body hypodermis from head to tail with a network-like endoplasmic reticulum pattern (Fig. 4). However, the signal of the mutant p*d11*^{ata}AGFPC was much weaker, although the profile did not appear to be much different. A pale green fluorescence was observed at the head and near the mouth region of the worms. This observation suggests that a greatly reduced level of product was made for the mutant *dpy-11* transgene with AUA as the start. This result is consistent with our second hypothesis that AUA was used as an inefficient start codon and the protein expression level was much reduced.

To have this second hypothesis further verified and to consider the possibility of the third alternative, the levels of the *dpy-11-gfp* fusion transcript and the DPY-11-GFP fusion proteins from these two transgenic animals were examined. The transmission rates of these two transgenic lines, p*d11*AGFPC and p*d11*^{ata}AGFPC, were between 40% and 50% within a very similar range from generation to generation. With the expression of an unrelated cuticular collagen gene *dpy-13* in these transgenic lines as an internal control, the RT-PCR analysis showed that the transgene RNA production levels of both transgenic lines were comparable (Fig. 5A). Thus, the mutation substituting AUG with AUA codon did not alter the transcriptional activity of the transgenes. This observation essentially eliminates our third hypothesis.

AUA can be recognized for translation initiation

To prove further that AUA could serve as a start codon, the molecular size of the two GFP-tagged proteins was evaluated with SDS–PAGE followed by a Western blot with GFP-specific antibody. Equal amounts of total worm extracts of these two transgenic lines were loaded into an SDS–PAGE and examined with anti-GFP antibody (Fig. 5B). In this Western blot, a 58 kDa band corresponding to the DPY-11::GFP fusion was detected in the lane of the p*d11*AGFPC transgenic line. The protein

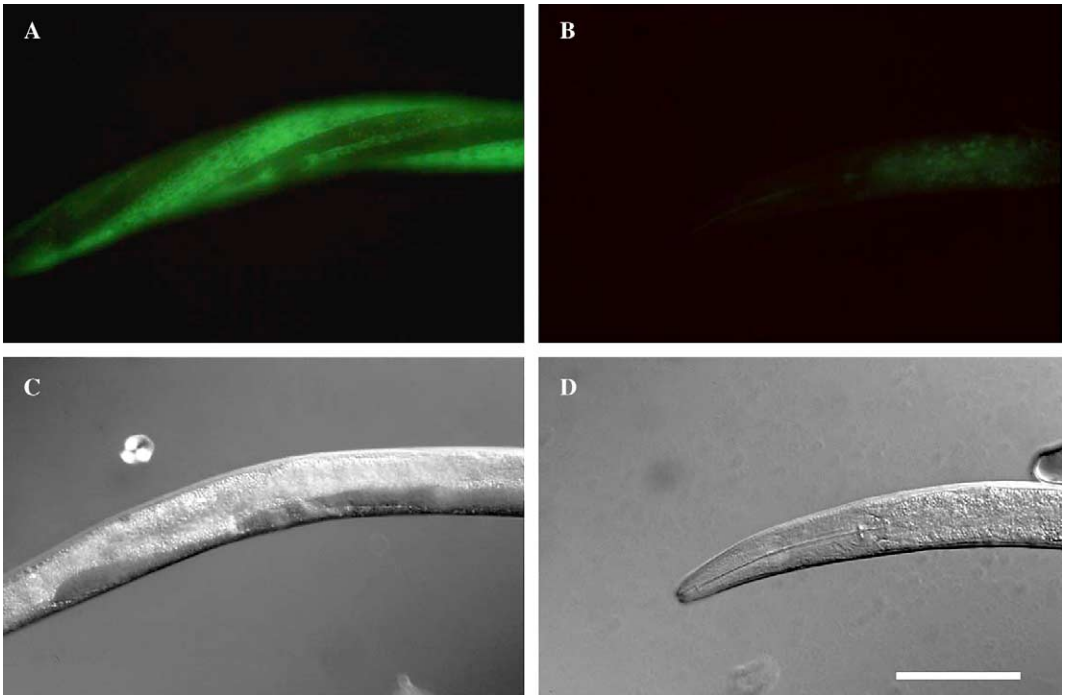

Fig. 4. *gfp* expression of (A) p*d11*AGFPC and (B) p*d11*^{ata}AGFPC transgenes in animals with wild-type genetic backgrounds and their DIC images shown in (C) and (D) respectively. The transgene with a mutant start codon displayed a weakened *gfp* signal in the body.

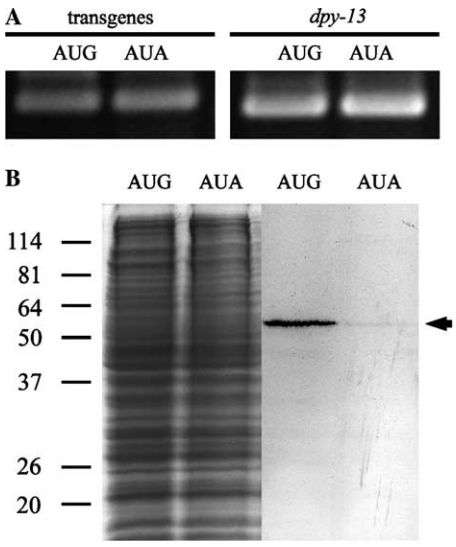

Fig. 5. Panel A, no difference in transcriptional activity of the two transgenes was observed by RT-PCR analysis. AUG and AUA stand for the products from the transgenic lines p*d11*AGFPC and p*d11*^{ata}AGFPC, respectively. The "transgenes" lanes represent the RT products from p*d11*AGFPC and p*d11*^{ata}AGFPC while "*dpy-13*" represents the control RT products from endogenous *dpy-13* transcripts. Panel B, SDS–PAGE and Western blot analysis of total proteins from transgenic lines p*d11*AGFPC (AUG) and p*d11*^{ata}AGFPC (AUA). Sixty-five microgram of total protein from each transgenic line was loaded to each line. A strong band and a weak band were detected with anti-GFP antibody in the AUG and AUA lanes, respectively (arrowhead).

size indicates the correct synthesis of the tagged protein, which is made at high level. On the other hand, the mutant p*d11*^{ata}AGFPC displayed a very weak band of the same molecular weight (Fig. 5B, arrow). Based on densitometric comparison, the level of the tagged mutant protein was at least 11 fold lower than that of the tagged wild-type DPY-11 product. The result is consistent with our observation of the GFP signal of these two transgenic lines under fluorescent microscopy (Fig. 4). Their products with identical molecular weight convincingly argue that the translation of these products started at the same position. This result supports our second hypothesis that a mutant AUA codon can be used for translation initiation for worms.

Discussion

With the *C. elegans* genome sequenced, the identification of genes is facilitated through various prediction programs [20]. The ORFs of genes are often confirmed by various functional assay and mutational studies. However, while many components of the translational machinery such as eIFs, elongation factors, ribosomal proteins, and numerous tRNAs were also identified in *C. elegans*, the exact mechanism determining the selection of translational initiation in *C. elegans* is largely unknown [21–24]. The prediction and interpretation of ORF to a great extent relies on the comparative information available from various studies of mammalian genes.

In *C. elegans*, the ORF of the *dpy-11* gene is confirmed with a full-length cDNA sequence followed by the fusion protein analysis [14]. An SL1 splice leader is trans-spliced to the RNA transcript in front of the authentic AUG codon. Thus, the 5′ AUG codon immediately behind the SL1 splice leader was predicted as the translation start. The property of this product having a signal peptide and being anchored on membranous structures within the cytoplasm of the hypodermis was verified by a reporter tag [14]. However, when this initiation AUG codon was mutated to AUA in this *dpy-11 s287* allele, the mutant phenotype surprisingly differed from that of a null allele (*e207*). Partial gene activity appeared to be retained. Our experimental results showed that this mutation at the initiation codon did not affect the transcription activity of the mutant locus. Neither did it alter the choice of translation initiation site. It only reduced the level of protein product, probably by attenuating the translation initiation efficiency. This reduction in DPY-11 abundance eventually leads to a loss-of-function phenotype with a mildly dumpy body and wild-type sensory rays in *s287* mutant animals.

Substitution of AUG with AUA as a functional start codon in eukaryotic genes has been reported before. For example, when the mouse dihydrofolate reductase gene initiating codon was mutated to AUA, the amount of protein product was reduced to 59% and 30% in vitro using rabbit reticulocyte and wheat germ lysate assays, respectively [25]. Although the size of in vitro synthesized product was not monitored in that study, the reduction of protein product was consistent with our observation that translation could still be initiated with a mutant start codon. In this study, the molecular weight of both the wild-type and mutant DPY-11 fusion protein products appeared identical in the Western blot. It is evident that the AUA codon could be used as a start site (Fig. 5B). The only impact this G to A transition is the efficiency of translation initiation. Since isoleucyl-tRNA with a perfect match with the AUA codon does not support translation initiation because of the side chain discrimination by the initiation factor 2 [26], we argue that the DPY-11 product from the *s287* mutant has a N-terminal methionine. The reduced DPY-11 fusion product level observed in our experiment is likely due to the inefficient incorporation of methionyl-tRNA to the AUA mutant start codon. Such an erroneous incorporation has been reported in an expression study of an archaebacterial ribosomal protein in bacteria [27]. It has also been shown in eukaryotes that the loss of this specificity is dependent on the weakened association of initiation factors, eIF2-β and -γ subunits [28]. Indeed, a few faint bands of 48, 35, 28, 26, and 20 kDa as opposed to the full length fusion protein of 58 kDa were detected in both AUG and AUA lanes in the same Western blot analysis. These smaller proteins account for 14–43% of full-length protein signal in the AUA lane. They are probably products of degradation since they are also present at a similar level in the AUG lane and they do not match the expected size of products should alternative AUG start sites be used. Moreover, none of these truncated products would be functional because all of them being smaller than 54 kDa would have the catalytic domain near the N-terminus destroyed or completely removed. In summary, with a majority of the purified products accounted for by the full-length protein in the AUA lane, it is clear that additional determining factors must be guiding the choice of the authentic AUG. For example, sequences adjacent to the AUG codon often play a crucial role in defining the choice of the start codon [3,5]. Statistical comparison also indicates a strong correlation between these adjacent sequences and the protein production level [29]. The surrounding contextual sequence around the authentic start codon of *dpy-11* mRNA would therefore be essential for the correct translation initiation. In the case of the *s287* mutation, although the primary AUG sequence was altered, the contextual sequence remains intact and may contain signature sequence or structural information sufficient to dictate this initiation site selection. As a result, the AUA codon embedded in this context was still favored over the other AUG sites at further downstream regions.

Concerning the choice of the AUG as a start codon, there are plenty of examples that AUA and UUG can also serve as a functional start codon in some mitochondrial, plastid, viral, and prokaryotic genes [6,10–12,30]. In eukaryotes, the use of AUG as the initiation codon remains the general rule. Except for rare cases where translation initiation start sites have been mutated from the AUG to AUA, essentially all genes have the translation starting at AUG [25,31]. In fact, in all these cases with the variant start codon, the initiation efficiency is often low as demonstrated by in vitro assay. Like most eukaryotic genes, wild-type *dpy-11* shows no exception to this general rule. On the other hand, we demonstrate in this report that for the *s287* allele, AUG is not an obligatory requirement for translation initiation. Our in vivo assay showed that a biologically functional protein at reduced abundance can be made with an AUA start. Moreover, the inefficiency of AUA for translation initiation is well correlated with the amount of stable protein produced as well as the severity of the mutant phenotype.

In our previous study, the mutant alleles, *e395*, *e431*, *s10*, and *s360*, have a similar phenotype as *s287* (14; Table 1). All these alleles have the mutations located in the transmembrane domain. Although the level of these mutant proteins was not analyzed, their ability to be retained on the membrane would certainly be affected [14]. As a result, only a limited amount of the functional products would be in the correct cellular compartment.

The quantification of these properly localized proteins cannot be ascertained easily. Yet, these mutant alleles have a strong impact on the body morphology but essentially no effect on the male tail sensory organ morphology. They all display wild-type sensory rays. Now that based on the phenotype of null animals rescued by a transgene with the *s287* mutation, such differential effects of the *dpy-11* on the body hypodermis and male tail can be addressed. With less than 10% of the wild-type DPY-11 protein level, the lumpy ray phenotype is completely reverted to wild-type while the body abnormality could be rescued only partially. The results presented here allow us to document the quantitative reduction of the protein level in this *s287* mutant, suggesting that the other transmembrane mutations probably had similar level of the mutant proteins retained at the endoplasmic reticulum. At the same time, the Western blot of the tagged products clearly defines the threshold requirement of *dpy-11* activity in the male tail. While wild-type body morphology cannot be maintained with the *dpy-11* activity below 50% of that in wild-type animals, male tail morphogenesis is not affected at all as long as around 10% of *dpy-11* function is present.

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