Int. J. Dev. Biol. 54: 1347-1353 (2010)
doi: 10.1387/ijdb.092862tt

THE INTERNATIONAL JOURNAL OF DEVELOPMENTAL BIOLOGY
www.intjdevbiol.com

Characterization of the 38 kDa protein lacking in gastrula-arrested mutant *Xenopus* embryos

TETSUYA S. TANAKA[1], FUMIKO NISHIUMI[2], TOHRU KOMIYA[2] and KOHJI IKENISHI*[,2]

[1]Department of Animal Sciences, Institute for Genomic Biology, University of Illinois at Urbana-Champaign, IL, USA and [2]Department of Biology, Graduate School of Science, Osaka City University, Sugimoto, Sumiyoshi, Osaka, Japan

ABSTRACT We have reported elsewhere that offspring from the No. 65 female of *Xenopus laevis* cleaved normally, but their development was arrested at the onset of gastrulation, like the *Ambystoma ova-deficient* (*o*) mutant, irrespective of mating with different wild-type males, and that an acidic, 38 kDa protein present in wild-type eggs was lacking in eggs of the female. In the current study, we first determined the partial amino acid sequence (VANLE) of one of the well-separated tryptic peptides from the protein, which was found in elongation factor 1 delta (Ef1δ) in *Xenopus*, and finally identified the protein as one of the Ef1δ isoforms, Ef1δ2, by peptide mass spectrometry. RT-PCR analyses for *Ef1δ2* and its close homolog *Ef1δ1* in wild-type oocytes and embryos demonstrated that both transcripts are maternal and *Ef1δ1* is present more abundantly than *Ef1δ2* throughout the stages examined. Importantly, the amount of the *Ef1δ2* transcript per embryo decreased gradually after gastrulation, in accordance with the gradual decrease of the 38 kDa protein per embryo reported in our earlier study. Because pharmacological inhibition of translation induces gastrulation arrest in wild-type embryos, it is reasonable to conclude that the mutant embryos arrest in development due to the lack of Ef1δ2 that is indispensable for translation. Thus, the present study provides the first molecular information on the cause of the gastrulation-defective mutation in Amphibia.

KEY WORDS: *elongation factor, gastrulation arrest, ova-deficient mutant, peptide mass spectrometry*

Gastrulation is an essential process for morphogenesis of embryos extending from coelenterates to vertebrates. As the mechanism of gastrulation is still not fully understood at the molecular level, it is useful to find mutants defective in gastrulation (Beetschen, 2001, Droin, 1992). A maternal-effect mutation in *Xenopus laevis* like the ova-deficient (*o*) mutation in *Ambystoma* (Humphrey, 1966) was reported in an earlier study (Ikenishi and Tsuzaki, 1988). All offspring from the female (designated as No. 65) cleave normally but arrest development at gastrulation, irrespective of mating to different wild-type males. By the analysis of 2 dimensional (2D) polyacrylamide gel electrophoresis (PAGE), it has been clarified that an acidic 38 kDa protein present in wild-type eggs is deficient in the mutant eggs. To understand the relationship between the absence of the protein and the developmental arrest, an antibody against the 38 kDa protein was raised and injected into fertilized wild-type eggs (Tanaka and Ikenishi, 2002). Almost all injected eggs cleaved normally but a majority arrested development at gastrulation, showing a phenocopy of the mutant embryos. In contrast, the majority of the control antibody-injected eggs gastrulated normally and developed further. These facts strongly indicate that the perturbation of the 38 kDa protein with the antibody induced the developmental arrest of the antibody-injected embryos at gastrulation.

To clarify the nature of the protein, we have characterized the 38 kDa protein by mass spectrometry. The sequence of oligopeptides independently obtained by enzymatic digestion of the protein separated by 2D PAGE revealed that the protein was elongation factor 1 delta 2 (Ef1δ2) in *Xenopus laevis* (Minella *et al.* 1996). The expression profile of *Ef1δ2* was examined, together with *Ef1δ1*, in wild-type oocytes and embryos by reverse-transcriptase polymerase chain reaction (RT-PCR). The profile of the *Ef1δ2* mRNA was comparable to that of the 38 kDa protein

Abbreviations used in this paper: Ef1, elongation factor 1; PAGE, polyacrylamide gel electrophoresis; RT-PCR, reverse transcriptase-polymerase chain reaction.

***Address correspondence to:** Kohji Ikenishi. Department of Biology, Graduate School of Science, Osaka City University, 3-3-138, Sugimoto, Sumiyoshi, Osaka 558-8585, Japan. Fax: +81-6-6605-3161. e-mail: ikenishi@sci.osaka-cu.ac.jp

Accepted: 7 July 2009. *Final author corrected PDF published online:* 28 June 2010. *Edited by:* Makoto Asashima.

ISSN: Online 1696-3547, Print 0214-6282

reported previously (Tanaka and Ikenishi, 2002), further indicating that the 38 kDa protein is *Ef1δ2*.

Results

Partial amino acid sequencing of tryptic peptide fragments and peptide mass spectrometry have identified the 38 kDa protein as eukaryotic translation elongation factor 1 delta 2

To unveil the identity of the 38 kDa protein, first, the 38 kDa protein from cleavage stage embryos separated by 2D PAGE was digested with trypsin in acrylamide gels. Tryptic peptides were fractionated by capillary HPLC and three abundant, well-separated peptides were used to determine partial amino acid sequence. When 5 N-terminal residues were sequenced from each of the peptides, Val-Ala-Asn-Leu-Glu (VANLE) was determined without ambiguity from one peptide, whereas the other two peptides generated mixed signals (Table 1).

When similar tryptic peptides from the 38 kDa protein were analyzed by peptide mass spectrometry, peptides having monoisotopic molecular masses of 850Da or larger were identified. The molecular mass profile of 34 peptides was subjected to searching a match in three databases (ProFound, Mascot and Aldente at ExPASy, http://ca.expasy.org/; Table 2). By summarizing the search results, a total of 14 different peptides out of 34 showed a match to *Xenopus laevis* eukaryotic elongation factor 1 delta 2 (Ef1δ2; Minella *et al.*, 1996). Peptide masses corresponding to predicted amino acid positions from 1 to 17, 60 to 72, 168 to 192 and 199 to 211 indicated a potential phosphorylation at one of the Ser or Thr sites (addition of 80Da), an oxidation of Met sites (addition of 16Da) or a combination of both (addition of 112Da; Table 2). The mapped peptides covered 57% of the predicted Ef1δ2 amino acid sequence with probability of 0.0091 or greater. Because predicted tryptic fragments from Ef1δ2 between the residues 88 to 167 had smaller molecular masses than 850Da except two at the residues 94 to 109 and 124 to 152, 13% of the predicted Ef1δ2 amino acid sequence could not be matched by this approach. Three predicted polypeptides near the C-terminal end (the residues 222-232, 233-244 and 245-259) and residues 124 to 152 of Ef1δ2 (26% of Ef1δ2) could not be matched to the rest of the peptides from the 38 kDa protein. Peptide mass tolerance larger than 1.2Da could not improve the search result. Perhaps the C-terminal end of Ef1δ2 might be modified by post-translational modification of proteins, which would prevent the polypeptide from being digested by trypsin. In support of this notion, Ef1δ2 exhibited different electrophoretic mobility from the molecular weight deduced from its predicted amino acid sequence. Further studies are required to determine whether the anomalous electrophoretic mobility of the Ef1δ2 protein in 2D PAGE is ascribed to its post-translational modification.

Ef1δ2 has been identified as a close homologue of Ef1δ1 (Morales *et al.*, 1992). Both Ef1δ1 and 2 are components of a larger functional protein complex with Ef1β and Ef1γ, which is a substrate of the Cdc2 kinase (Mulner-Lorillon *et al.*, 1994) and acts as a guanine-nucleotide exchange protein (Minella *et al.*, 1996). As amino acid sequences of Ef1δ1 and 2 share 85.5% of identity and have similar molecular weight (34k and 36kDa, respectively; Minella *et al.*, 1996), next we have asked if there is a possibility that the 38 kDa protein is Ef1δ1. For the following three reasons, we have excluded this possibility. First, as indicated in Table 2, 8 out of 14 peptides matched to Ef1δ2 were expected to be generated commonly from Ef1δ1. However, none of the 6 peptides that signify Ef1δ1 (double asterisks in Table 2) matched to the rest of the observed peptide masses, whereas 5 out of the 6 peptides that signify Ef1δ2 had matched to the observed peptide masses (p=0.015 by Fisher's exact probability test). Second, based on the independent attempt to sequence tryptic peptides from the 38 kDa protein spot as described above (Table 1), we could easily identify the peptide sequence VANLE and a deduced sequence from Peptide 3 on Table 1, Thr-Val-Gln-Met-Asp (TVQMD), in two (or three with a modified methionine residue; Table 2) of the predicted peptides from both Ef1δ1 and 2. However, the other deduced amino acid sequence from Peptide 2 on Table 1, Ser-Ser-Asn-Ser-Gln (SSNSQ), was found only in one of the predicted peptides from Ef1δ2. Because the corresponding predicted peptide (1996.07Da) from Ef1δ1 contains SSKSQ, which could be cleaved by trypsin at the C-terminal end of lysine (K) and generated 320.17Da and 1693.91Da fragments, it is less likely that these predicted peptides from Ef1δ1 can be matched to the analyzed peptides from the 38 kDa protein. Although the unmatched observed peptide mass of 1698.998Da from the 38 kDa protein was closer to the Ef1δ1 peptide of 1693.91Da, the difference of 5.088Da was too large for them to be matched under the current condition. Even if they were matched, the other 5 signature peptides of Ef1δ1 still need to be matched to the observed peptide masses of the 38 kDa protein. Finally, as will be described below, the temporal expression pattern of the *Ef1δ1* transcript showed discrepancy from that of the 38 kDa protein (compare Fig. 1B and Fig. 2a in Tanaka and Ikenishi, 2002). Thus, we have concluded that the 38 kDa protein is not Ef1δ1, but Ef1δ2.

TABLE 1

RESULTS OF AMINO ACID SEQUENCING OF N-TERMINAL ENDS OF TRYPTIC PEPTIDES

	Peptide 1	Peptide 2	Peptide 3
1st / candidates	**V**	**L** S	L V T S
2nd	**A**	**E** A V S	A V E G
3rd	**N**	**N** D P	Q I N E
4th	**L**	**S** L	M L
5th	**E**	**Q** V	D N E

Amino acid residues sequenced with higher confidence are shown in bold font.

Expression profiles of the **Ef1δ1** *and* **Ef1δ2** *transcripts in oocytes and embryos further validate that Ef1δ2 is the 38 kDa protein*

Previously, we showed by immunoblotting using a specific monoclonal antibody against the 38 kDa protein that the protein was present in oocytes (stages I-III mixed, IV, V and VI), unfertilized eggs, fertilized eggs, and embryos at cleavage through tadpole stages examined (see Fig. 2a in Tanaka and Ikenishi, 2002). Therefore, we have asked if *Ef1δ1* or *Ef1δ2* transcripts show similar temporal expression patterns in oocytes, eggs and embryos. Although *Ef1δ1* and *Ef1δ2* are identical through 93% of their sequence, we managed to detect products specific to each gene by semi-quantitative RT-PCR (see Materials and Methods).

Both *Ef1δ1* and *2* transcripts were detected in oocytes of all stages examined (stages I-III, IV, V and VI) and unfertilized eggs

TABLE 2

TRYPTIC PEPTIDE MASS SPECTROMETRY OF THE 38 kDa PROTEIN

Observed Peptide mass[a]	Matched mass[b] (calculated, ± 1.2 Da tolerance)	Peptide sequence[c] (position in Ef1δ2)	Predicted peptide mass[d] (> 850 Da) Ef1δ2 (260 a.a) NP 001084375	Predicted peptide mass[d] (> 850 Da) Ef1δ1 (265 a.a.) NP 001081523	Corresponding peptide sequence from Ef1δ1[e]
858.468					
863.272		DLQSAISK (76-83)	*861.47*	*861.47*	
877.032					
884.288					
892.999					
909.404					
945.554	944.57	LVPVGYGIK (212-220)	**945.58**	**945.58**	
962.433					
1023.569					
1059.471	1058.47	*YK* YDDAER (16-23)	**1058.47**	1030.46**	*YK* YDDAEK
1073.653	1072.66	LVPVGYGIK *K* (212-221)	**1072.66**	**1072.66**	
1205.616					
			1289.64 (222-232)	1289.64	
			1346.70 (233-244)	1346.70	
1356.794					
1372.774	1371.77	DLQSAISK *LEIR* (76-87)	**1371.77**	1345.72**	DLQSAISK *LESR*
1407.678	1406.69	**TVQMD**GLVWGSSK (199-211)	**1407.69**	**1407.69**	
1423.672	1422.68	TVQM*DGLVWGSSK (199-211)			
1509.751	1508.76	**VANLE**QENQSLHK (60-72)	**1509.77**	**1509.77**	
1588.808	1588.72	**VANLE**QENQS*LHK (60-72)			
1622.866	1621.88	**SSNSQ**KPAAAPQPVIK (94-109)	**1622.89**	1996.07**	*SSK* SQKPAAASQPAIEVAAR
1623.870					
1698.998					
			1745.84 (245-259)	1745.84	
1755.841					
1879.961					
2026.014					
2042.893					
2062.969	2061.99	SSILLDVKPWDDETDMAK (175-192)	**2063.00**	**2063.00**	
2081.021	2079.96	M*SAS*VIATEQVWLDK *YK* (1-17)	***1968.00***	2058.05**	MSAFVITTEQVWLDK *YK*
2629.212					
2863.296					
2868.295	2867.40	*KPGVIAK* S*SILLDVKPWDDETDM*AK (168-192)	***2755.45***	***2755.45***	
			3189.19 (124-152)		
				3402.26**	
3643.370					
3660.536	3659.61	QYYENLSGGSSPNNPHNSPQSAAPSNSGDGSELAAR (24-59)	**3660.62**		
3661.502	3659.61	QYYENLSGGSSPNNPHNSPQSAAPSNSGDGSELAAR (24-59)			
3784.786					
				3896.77**	

a: Observed peptide masses are highlighted in bold when the mass matches with a predicted mass, and by open squares when the match is uniquely found among the peptides from Ef1δ2. Each row represents a unique peptide mass.

b: The list of matched peptide masses was created by combining the search results in Mascot and ProFound databases via ExPASy. Some of the peptides are shown twice due to the potential amino acid modification. Although the peptide DLQSAISK was not identified as a match by the database search at tolerance of 1.2Da, its predicted mass shows only 1.8Da difference from the closest mass observed.

c: The peptide sequence whose mass shows a match with an observed mass is listed as single letters. The position of the amino acid in Ef1δ2 is indicated in parentheses. Five amino acid residues sequenced independently are shown in bold. Asterisks (*) indicate an oxidized methionine (M) or a phosphorylated serine (S), threonine (T) or tyrosine (Y). The phosphorylated T, S or Y residue is marked based on the probability by NetPhosK 1.0 and NetPhos 2.0 servers at ExPASy. The italicized letters indicate amended smaller tryptic peptide fragments when trypsin misses one recognition site, which is the C-terminal end of Lysine (K).

d: Tryptic peptide masses are predicted for Ef1δ1 and Ef1δ2 by PeptideMass at ExPASy. Each row represents a unique peptide mass. RefSeq numbers used to obtain the peptide mass are presented. The predicted mass for peptides smaller than 850Da is not shown because the mass spectrum was measured for peptides larger than 850Da with higher confidence. Matched peptide masses to the observed ones are shown in bold. Three types of predicted peptides were italicized because (1) the peptides of 861.47Da were not considered as a match whereas their predicted mass was relatively closer to the observed peptide mass of 863.272Da, and (2) the peptides of 1968.00Da and 2755.45Da were considered as matches when modifications in amino acid residues were taken into account. For unmatched peptide masses, position of the amino acid in Ef1δ2 is indicated in parentheses. Double asterisks (**) indicate the predicted unique peptide mass from Ef1δ1. Five out of 6 unique peptides larger than 850Da from Ef1δ2 matched with observed peptides, whereas none of unique peptides from Ef1δ1 matched with observed peptides (p=0.015). The matched peptides cover 57% of the predicted Ef1δ2 amino acid sequence.

e: The peptide sequence from Ef1δ1 that is closely relative to the matched peptides from Ef1δ2 is shown. Amino acid substitution is underlined. The italicized letters indicate amended smaller tryptic peptide fragments.

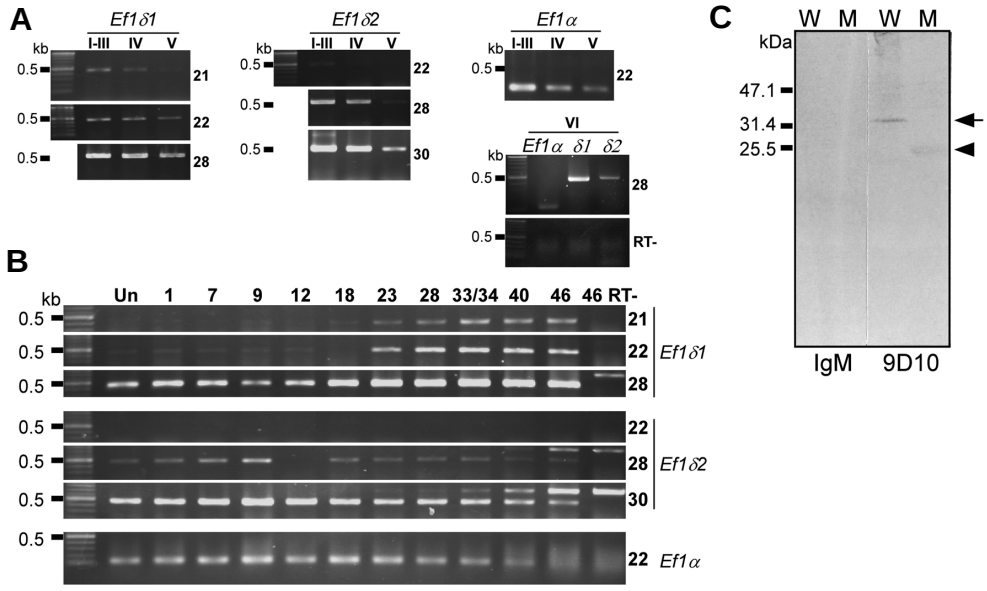

Fig. 1. Temporal expression profiles of the Ef1δ1 and Ef1δ2 transcripts and immunological detection of the 38 kDa protein. (A) *Primer pairs for Ef1δ1 (left), Ef1δ2 (middle) and Ef1α (right, top) are used to perform semi-quantitative (sq) RT-PCR with cDNAs prepared from oocytes at stages I-III mixed, IV, and V (Dumont, 1972) as indicated on top of the images. Likewise, the results of sqRT-PCR with cDNA from and RT-negative reaction for stage VI oocytes (right, bottom) are shown. Ef1α is a loading control. The number of PCR cycles is indicated on the right of each image. The position of 0.5 kb is indicated on the left.* **(B)** *Primer pairs for Ef1δ1 (top), Ef1δ2 (middle) and Ef1α (bottom) are used to perform sqRT-PCR with cDNAs prepared from unfertilized (Un) or fertilized eggs (1), morulae (7), late blastulae (9), late gastrulae (12), neurulae (18 and 23), tail-bud stage embryos (28), hatched tadpoles (33/34), swimming tadpoles (40) and feeding tadpoles (46) as indicated on top of the panel. Ef1α is a loading control. The number of PCR cycles is indicated on the right of each image. RT-negative reaction for the sample of feeding tadpoles (46 RT-) is presented. Although the RNA samples of stages 33/34, 40 and 46 are obviously contaminated with the genomic DNA, it does not interfere with our interpretation of the results (see text for details). The position of 0.5 kb is indicated on the left.* **(C)** *Immunoblot with the monoclonal antibody 9D10 that specifically reacts with the 38 kDa protein (Tanaka and Ikenishi, 2002). The protein samples from wild-type (W) and mutant (M) embryos were processed for immunoblotting with 9D10 and IgM. 9D10 recognizes a 38 kDa band in the wild-type and a 25 kDa band in the mutant. IgM does not react with any band in either sample.*

by RT-PCR (Figs. 1 A,B). Accordingly, *Ef1δ1* and *Ef1δ2* are maternal transcripts and the abundance of the transcripts in oocytes, ranked from highest to lowest, was *Ef1α*, *Ef1δ1* and *Ef1δ2*. After fertilization, *Ef1δ1* was detected constitutively until the late gastrula stage (stage 12), upregulated at the neurula stage (stage 18-23) and reached the plateau expression level after hatching (stage 33/34) (Fig. 1B). At the tadpole stage, *Ef1δ1* was the major elongation factor among *Ef1δ1*, *Ef1δ2* and *Ef1α*. On the other hand, the amount of the *Ef1δ2* transcript increased constantly by the late blastula stage (stage 9) and declined at stage 12. It became less abundant from stage 18 onward and was rare at the tadpole stage (stages 40 and 46). In addition, a larger PCR product than that of *Ef1δ2* was amplified in the samples from stages 33/34 to 46 (Fig. 1B). It was also observed in the sample of stage 46 without RT (Fig. 1B, the lane "46 RT-"). The product was derived from contaminated genomic DNA, because by direct sequencing it had segments of the sequence corresponding to the *Ef1δ2* mRNA separated by an intervening sequence with GTAAGT at the 5' end, and TACTAAC that falls into a consensus sequence, YNYURAY, of the branch site, followed by a polypyrimidine tract and AG at the 3' end (Horowitz and Krainer, 1994; Genbank accession numbers GQ379233 and GQ379234). Theoretically, when two templates that can be amplified with the same set of primers that differ in length are present in the same PCR, a shorter product will be amplified more efficiently. By contrast, the amount of the larger PCR product derived from the genomic DNA increased from stage 33/34 onwards, whereas the consistent decrease of the *Ef1δ2* expression level was observed. Thus, this result reflects the abundance of the initial *Ef1δ2* transcript level in the PCR and the contamination of the genomic DNA itself does not interfere with our interpretation of the results obtained.

Finally, we compared the expression profiles of the *Ef1δ1* and *Ef1δ2* transcripts (Fig. 1B) with that of the 38 kDa protein detected by the specific monoclonal antibody, 9D10 (Tanaka and Ikenishi, 2002). The abundance of the *Ef1δ2* transcript that was present throughout the cleavage stages decreased gradually after gastrulation, in contrast with the significant increase of the copy number of the *Ef1δ1* transcript from the neurula stage onward (Fig. 1B). On the other hand, the 38 kDa protein that was present at a constant level by the late blastula stage (stage 9) decreased its level steadily from the mid-tailbud stage (stage 28) onward (see Fig. 2a in Tanaka and Ikenishi, 2002). These results demonstrate that the temporal change in the amount of the *Ef1δ2* transcript was comparable to that of the 38 kDa protein during embryonic development.

In connection with the absence of the 38 kDa protein in the mutant embryos, immunoblotting with 9D10 or a control non-specific IgM antibody was performed to the protein samples from cleavage stage wild-type and mutant embryos. The 9D10 antibody reacted with the 38 kDa band in the sample from wild-type embryos and with a 25kDa band in the sample from the mutant embryos, whereas the control antibody did not react at all in both samples (Fig. 1C). The possible origin of the 25kDa band is discussed below (see Fig. 2).

Discussion

In the present study, we have shown the first molecular evidence implicating a specific gene in gastrulation arrest observed in the ova-deficient mutant in *Xenopus laevis*. By analyzing tryptic peptide fragments originated from the 38 kDa protein lacking in the mutant embryos, we could determine the protein to be eukaryotic translation elongation factor 1 delta 2 (Ef1δ2), owing to the accumulation of data for *Xenopus* genes. This result

has been further validated by the similarity of the temporal expression profiles between the *Ef1δ2* transcript and the 38 kDa protein detected by the specific antibody (Tanaka and Ikenishi, 2002). That is, after gastrulation, the amount of the *Ef1δ2* transcript per embryo decreased gradually, in accordance with gradual decrease of the amount of the 38 kDa protein per embryo shown in the previous study (Tanaka and Ikenishi, 2002). By contrast, the amount of the *Ef1δ1* transcript per embryo increased significantly with development after gastrulation, showing discrepancy from that of the 38 kDa protein (compare Fig. 1B and Fig. 2a in Tanaka and Ikenishi, 2002). It is well known that wild-type embryos arrest development at the onset of gastrulation when they are treated with cycloheximide or an inhibitor in protein synthesis prior to the MBT (Hensey and Gautier, 1997). These arrested embryos exhibit characteristic features of apoptosis including fragmentation of nuclei (Hensey and Gautier, 1997), which is another phenotype observed in the mutant embryos from the No.65 female (Ikenishi and Tsuzaki, 1988). Therefore, it is likely that the mutant embryos lacking Ef1δ2 could not initiate *de novo* protein synthesis, resulting in the developmental arrest at gastrulation, possibly by activation of apoptosis. Similarly, the mutant phenocopy induced in wild-type embryos by injecting the 9D10 antibody (Tanaka and Ikenishi, 2002) might be due to the inhibition of *de novo* protein synthesis via the perturbation of the Ef1δ2 function. However, the phenocopy was not induced by microinjection of antisense morpholinos against the *Ef1δ2* transcript into fertilized wild-type eggs (data not shown). Perhaps the amount of the Ef1δ2 protein present already in the fertilized eggs might be sufficient enough to complete gastrulation.

It is interesting to note that Ef1δ2 exhibits different electrophoretic mobility from the molecular weight deduced from its predicted amino acid sequence. The predicted primary amino acid sequence of Ef1δ2 translated from its cDNA has the molecular weight of 28.63kDa at a pI of 4.6. On the other hand, Ef1δ2 has been identified as a component of 36kDa with a specific polyclonal antibody against a guanine-nucleotide exchange protein complex that is a substrate of the Cdc2 kinase (Mulner-Lorillon *et al.*, 1994). Anomalous electrophoretic mobility of proteins in PAGE has been ascribed to post-translational modifications, amino acid composition (very hydrophobic, basic or acidic), and transcription or translation attenuation (Klenova *et al.*, 1997). According to database search, Ef1δ2 has two potential Small Ubiquitin-like Modifier (SUMO) modification sites at the 123rd and 192nd Lysine (K) residues, which typically exhibit the consensus sequence of hKxE, where h and x are a hydrophobic and any amino acid, respectively (Panse *et al.*, 2004). Because SUMO is a 100-residue protein (Panse *et al.*, 2004), the molecular weight of a protein ligated with one SUMO will be increased by about 10kDa. Interestingly, the affinity chromatography-based proteomic approach in yeast has identified that eukaryotic translation elongation factor 2 encoded by the *Eft2* gene is one of the SUMO targets (Panse *et al.*, 2004). Unlike ubiquitylation, SUMOylation of target proteins does not lead to proteasomal degradation but can affect diverse functions of the protein, such as subcellular localization, protein/DNA interaction, or enzymatic activity (Panse *et al.*, 2004).

By immunoblotting with the 9D10 antibody specific to the 38 kDa protein in wild-type embryos, a faint band with the molecular weight of about 25kDa was detected in the sample from the mutant embryos (Fig. 1C). The difference of the molecular weight between the 25kDa band and without post-translational modification is about 4kDa, which corresponds to 30 to 40 amino acids. Therefore, a plausible scenario to explain the lack of the 38 kDa protein in the mutant embryos may be frame shift at the C-terminal end of Ef1δ2 that causes not only premature termination of translation but also mutation in the site of post-translational protein modification. In fact, insertion of a single nucleotide within the codons encoding one of the putative SUMOylation sites (GCC AAG CTG GAG) or the codon of K192 in Ef1δ2 (AAG; Minella *et al.*, 1996) has potential to translate a polypeptide that supports our result of immunoblotting (Figs. 1C and 2). Unfortunately, however, it is impossible to validate our model in the mutant embryos directly because the mutant female was dead more than 20 years

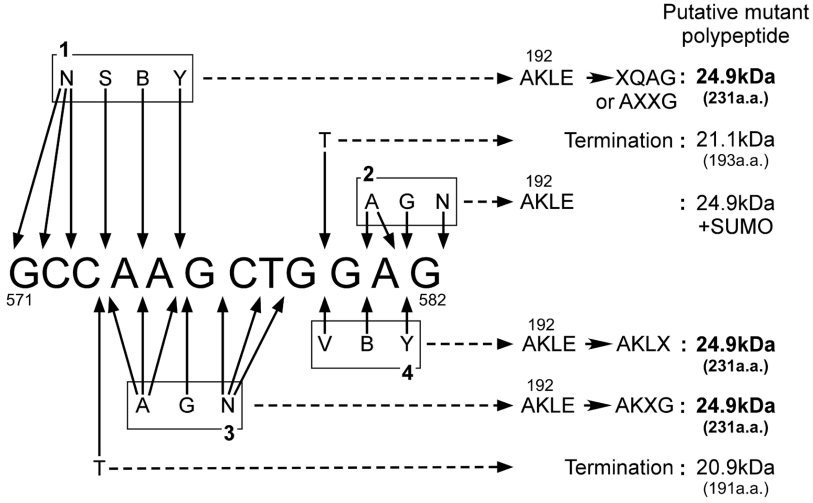

Fig. 2. A model to explain the lack of the 38 kDa protein in the mutant embryos. *Possible manners of frame shift in Ef1δ2 are shown by insertion of a single nucleotide within a sequence motif, GCC AAG CTG GAG (between 571bp and 582bp from ATG), which is translated into AKLE in amino acid sequence, a predicted SUMOylation site. Insertion of a nucleotide as indicated in the box 1 will substitute K192 in AKLE with other amino acids (XQAG or AXXG). Insertion of a nucleotide as indicated in the box 3 and 4 will leave K192 intact but change the putative SUMOylation site (AKLE) into different contexts (AKXG or AKLX; in the latter case X is other amino acid than E). In either way, these mutations will terminate translation prematurely to generate a polypeptide of 231 amino acids with the predicted molecular weight of 24.9kDa (bolded), which shows consistency with the result of immunoblotting in Fig. 1C. Insertion of a nucleotide as indicated in the box 2 will leave K192 intact as well as the putative SUMOylation site (AKLE), such that this will generate a polypeptide of 231 amino acids modified by SUMOylation. Insertion of T as indicated will terminate translation prematurely to generate polypeptides with the molecular weight shorter than 24.9 kDa. Deletion of any single nucleotide within the codons encoding the motif of AKLE will not terminate translation at the original site but translate a polypeptide of 317 amino acids, no matter how the deletion changes the predicted SUMOylation site. B: C or G or T, N: A or C or G or T, S: C or G, V: A or C or G, Y: C or T.*

ago.

It was the first in Amphibia that gastrulation arrest of the *o* mutant embryos in *Ambystoma* was restored by microinjection of the unidentified protein component from wild-type eggs (Briggs and Justus, 1968). The present study clarifies that the 38 kDa protein, which is lacking in the maternal-effect mutant embryos in *Xenopus* showing a similar phenotype, is the Ef1δ2 protein. It will be interesting to study whether orthologs of Ef1δ2 will play an evolutionarily conserved role in gastrulation of higher vertebrates, such as mouse and human.

Materials and Methods

The African clawed frog *Xenopus laevis* was used to provide oocytes, eggs and embryos according to the approved protocol to K. I. by the institutional animal care and use committee at the Osaka City University. Collection of wild-type oocytes, eggs and embryos and mutant embryos was done essentially as described previously (Ikenishi and Tsuzaki, 1988, Tanaka and Ikenishi, 2002). Oocytes and embryos were staged after Dumont (1972) and Nieuwkoop and Faber (1967), respectively. No embryo collection from live *Xenopus laevis* has taken place at the University of Illinois. Unless otherwise noted, chemical reagents were purchased from Wako pure Chemical Industries, Osaka, Japan.

Preparation and characterization of the 38 kDa protein

Two-dimensional (2D) PAGE with protein samples (400~600 μg per gel) from cleavage stage embryos (stage 2-7) was carried out essentially as described previously (Ikenishi and Tsuzaki, 1988, Tanaka and Ikenishi, 2002). After staining with 0.1% Coomassie Brilliant Blue-R250 (Sigma-Aldrich, St. Louis, MO, USA), 50% methanol, 10% acetic acid for 30 min at 22-24ºC, gels were destained with 5% methanol, 7.5% acetic acid overnight at 22-24ºC with agitation. After a brief rinse with distilled water, a spot of the 38 kDa protein, another major protein spot having a similar molecular weight (positive control) and a similar-sized gel piece of protein free area (negative control) were excised with a scalpel from the gels. Gel pieces of those spots (2 x 3 x1 mm^3 each) were pooled separately in a siliconized tube. After boiling for 5 min, the gel pieces in the tubes were quickly chilled on ice, destained 3 times with 100mM NH_4HCO_3 (pH 8.9), 50% CH_3CN at 22-24ºC for 30 min each with agitation and dried in a SpeedVac for 30 min. The resultant gel pieces were rehydrated with a solution of sequence grade trypsin (Promega Co., Madison, WI, USA; reconstituted in 1 mM HCl at 0.1 μg/μl, 0.1 μg/gel piece) containing 200 mM NH_4HCO_3 (pH 8.9) and 0.2% Tween 20, and incubated at 37ºC overnight (Hellman *et al.*, 1995; Rosenfeld *et al.*, 1992). The reaction was terminated by adding 10% trifluoroacetic acid (TFA) at 1/10 in volume of the reaction mixture (the final concentration was 1%) and the tryptic peptides were eluted 3 times into 60% CH_3CN, 0.1% TFA at 30ºC for 40 min each with agitation. After reduction to the appropriate volume by evaporation, these samples were used for determining the peptide sequence (Biologica Co., Nagoya, Japan) and for peptide mass spectrometry (MALDI-TOF mass spectrometry at the Proteomics and Mass Spectrometry Unit of the National Institute on Aging, NIH, Baltimore, MD, USA). We estimated that 10 pieces of the 38 kDa protein spots separated by 2D PAGE from a total of 6 mg of the protein sample of cleavage stage embryos (stages 2-7) were equivalent to about 100 pmol of the 38 kDa protein. Protein identification based on the monoisotopic peptide masses was done by searching databases such as ProFound, Mascot and Aldente at ExPASy (http://ca.expasy.org/) by defining the taxon as amphibian or *Xenopus laevis*.

Semi-quantitative RT-PCR

RNA extraction and first strand cDNA synthesis for polymerase chain reaction (PCR) were done essentially as described previously (Ikenishi and Tanaka, 2000; Tanaka *et al.*, 2008; Tanaka *et al.*, 2006). Briefly, 5 oocytes at stages IV, V and VI, 5 unfertilized eggs or 5 embryos (stages 1, 7, 9, 12, 18, 23, 28, 33/34, 40 and 46) were pooled for RNA extraction. For oocytes at stages I-III, we pooled 5 oocytes at stages I, II and III together because of the smaller volume of the oocyte at those stages. Quality of extracted RNA was validated with the recovery of 28S and 18S ribosomal RNA on the ethidium bromide-stained agarose gel by electrophoresis. RNA equivalent to one-half of an oocyte (at stages IV-VI), an egg or an embryo at each stage and 1.5 oocytes at stages I-III (1/10 of extracted RNA) was used for cDNA synthesis with SuperScript II (Invitrogen Co., Carlsbad, CA, USA). Likewise, cDNA synthesis without reverse-transcriptase (RT) was also performed as negative controls. One-fiftieth of each cDNA was used as a template for the following PCR with Phusion DNA polymerase (New England Biolabs Inc., Ipswich, MA, USA). The primer pairs used are as follows:

for *Ef1δ1* (469bp),
forward, GGTACAAGTCACACCCGCTGC,
reverse, AGAGTCCAGCAAGCCAGGG, annealed at 65ºC;
for *Ef1δ2* (484bp),
forward, GGGAGGATGATGATGACGATATCG,
reverse, CACCCAGTCGGTACATAAACGAG, annealed at 60ºC. The reverse primer of *Ef1δ2* was designed from part of the 3' UTR specific to *Ef1δ2*. The PCR product for *Ef1δ1* but not *Ef1δ2* has one BamHI site that gives rise to two fragments of 116 and 353 bp long, which is used to validate the *Ef1δ1* PCR product. To further validate specificity of the PCR products, they were directly sequenced by using forward primers for *Ef1δ1* or *Ef1d2*. Similarly, PCR products observed in the sample of stage 46 without RT (Fig. 1B) were sequenced (Genbank accession numbers GQ379233 and GQ379234). *Ef1α* was used as a loading control (Krieg and Melton, 1985).

Immunoblotting

Immunoblotting of protein samples of wild-type and mutant embryos with the antibody specific to the 38 kDa protein, 9D10, or a control IgM antibody was carried out as described previously (Tanaka and Ikenishi, 2002).

Acknowledgements

We would like to thank Dr. Kazuo Ito, Miss Yoko Tanigawa and Dr. Cheol-Soon Lee for technical assistance and Dr. David Miller for critical reading of this manuscript. Thanks are also expressed to Dr. Mitsuru Furusawa for intellectual interaction and encouragement. TST was an awardee of the research fellowship for young scientists of the Japan Society for the Promotion of Science (JSPS). This study was supported in part by ERATO project (KI) and the departmental startup fund including the USDA Cooperative State Research, Education and Extension Service, Hatch project number ILLU-538-323 (TST).

References

BEETSCHEN, J.C. (2001). Amphibian gastrulation: history and evolution of a 125 year-old concept. *Int J Dev Biol* 45: 771-795.

BRIGGS, R. and JUSTUS, J.T. (1968). Partial characterization of the component from normal eggs which corrects the maternal effect of gene o in the Mexican axolotl (Ambystoma mexicanum). *J Exp Zool* 167: 105-116.

DROIN, A. (1992). The developmental mutants of *Xenopus*. *Int J Dev Biol* 36: 455-464.

DUMONT, J.N. (1972). Oogenesis in *Xenopus laevis* (Daudin). I. Stages of oocyte development in laboratory maintained animals. *J Morphol* 136: 153-179.

HELLMAN, U., WERNSTEDT, C., GONEZ, J. and HELDIN, C.H. (1995). Improvement of an "In-Gel" Digestion Procedure for the Micropreparation of Internal Protein Fragments for Amino Acid Sequencing. *Anal Biochem* 224: 451-455.

HENSEY, C. and GAUTIER, J. (1997). A developmental timer that regulates apoptosis at the onset of gastrulation. *Mech Dev* 69: 183-195.

HOROWITZ, D.S. and KRAINER, A.R. (1994). Mechanisms for selecting 5' splice

sites in mammalian pre-mRNA splicing. *Trends Genet* 10: 100-106.

HUMPHREY, R.R. (1966). A recessive factor (o, for ova deficient) determining a complex of abnormalities in the Mexican axolotl (Ambystoma mexicanum). *Dev Biol* 13: 57-76.

IKENISHI, K. and TANAKA, T.S. (2000). Spatio-temporal expression of *Xenopus* vasa homolog, XVLG1, in oocytes and embryos: the presence of XVLG1 RNA in somatic cells as well as germline cells. *Dev Growth Differ* 42: 95-103.

IKENISHI, K. and TSUZAKI, Y. (1988). A possible maternal-effect mutant of *Xenopus laevis* I. Cytological and biochemical analyses of the unfertilized eggs and embryos. *Dev Biol* 125: 458-461.

KLENOVA, E.M., NICOLAS, R.H., U, S., CARNE, A.F., LEE, R.E., LOBANENKOV, V.V. and GOODWIN, G.H. (1997). Molecular weight abnormalities of the CTCF transcription factor: CTCF migrates aberrantly in SDS-PAGE and the size of the expressed protein is affected by the UTRs and sequences within the coding region of the CTCF gene. *Nucleic Acids Res* 25: 466-474.

KRIEG, P.A. and MELTON, D.A. (1985). Developmental regulation of a gastrula-specific gene injected into fertilized *Xenopus* eggs. *EMBO J* 4: 3463-3471.

MINELLA, O., MULNER-LORILLON, O., POULHE, R., BELLE, R. and CORMIER, P. (1996). The guanine-nucleotide-exchange complex (EF-1 beta gamma delta) of elongation factor-1 contains two similar leucine-zipper proteins EF-1 delta, p34 encoded by EF-1 delta 1 and p36 encoded by EF-1 delta 2. *Eur J Biochem* 237: 685-690.

MORALES, J., CORMIER, P., MULNER-LORILLON, O., POULHE, R. and BELLE, R. (1992). Molecular cloning of a new guanine nucleotide-exchange protein, EF1 delta. *Nucleic Acids Res* 20: 4091.

MULNER-LORILLON, O., MINELLA, O., CORMIER, P., CAPONY, J.P., CAVADORE, J.C., MORALES, J., POULHE, R. and BELLE, R. (1994). Elongation factor EF-1 delta, a new target for maturation-promoting factor in *Xenopus* oocytes. *J Biol Chem* 269: 20201-20207.

NIEUWKOOP, P.D. and FABER, J. (1967). *Normal table of Xenopus laevis.* North-Holland Publishing, Amsterdam.

PANSE, V.G., HARDELAND, U., WERNER, T., KUSTER, B. and HURT, E. (2004). A Proteome-wide Approach Identifies Sumoylated Substrate Proteins in Yeast. *J. Biol. Chem.* 279: 41346-41351.

ROSENFELD, J., CAPDEVIELLE, J., GUILLEMOT, J.C. and FERRARA, P. (1992). In-gel digestion of proteins for internal sequence analysis after one- or two-dimensional gel electrophoresis. *Analytical Biochemistry* 203: 173-179.

TANAKA, T.S., DAVEY, R.E., LAN, Q., ZANDSTRA, P.W. and STANFORD, W.L. (2008). Development of a gene trap vector with a highly-sensitive fluorescent protein reporter system aiming for the real-time single cell expression profiling. *Genesis* 46: 347-356.

TANAKA, T.S. and IKENISHI, K. (2002). Possible role of the 38 kDa protein, lacking in the gastrula-arrested *Xenopus* mutant, in gastrulation. *Dev Growth Differ* 44: 23-33.

TANAKA, T.S., LOPEZ DE SILANES, I., SHAROVA, L.V., AKUTSU, H., YOSHIKAWA, T., AMANO, H., YAMANAKA, S., GOROSPE, M. and KO, M.S. (2006). Esg1, expressed exclusively in preimplantation embryos, germline, and embryonic stem cells, is a putative RNA-binding protein with broad RNA targets. *Dev Growth Differ* 48: 381-390.