On a Potential Global Role for Vitamin K-dependent \( \gamma \)-Carboxylation in Animal Systems

EVIDENCE FOR A \( \gamma \)-GLUTAMYL CARBOXYLASE IN DROSOPHILA\(^*\)

Craig S. Walker‡, Reshma P. Shetty‡§, Kathleen Clark‡, Sandra G. Kazuko¶, Anthea Letsou¶, Baldemero M. Olivera‡, and Pradip K. Bandyopadhyay‡¶

From the Departments of \( \ddagger \)Biology and \( \ddagger \)Human Genetics, University of Utah, Salt Lake City, Utah 84112-0840

The vitamin K-dependent \( \gamma \)-carboxylation of glutamate to \( \gamma \)-carboxyglutamate was originally well characterized in the mammalian blood clotting cascade. \( \gamma \)-Carboxyglutamate has also been found in a number of other mammalian proteins and in neuropeptides from the venoms of marine snails belonging to the genus Conus, suggesting wider prevalence of \( \gamma \)-carboxylation. We demonstrate that an open reading frame from a Drosophila melanogaster cDNA clone encodes a protein with vitamin K-dependent \( \gamma \)-carboxylase activity. The open reading frame, 670 amino acids in length, is truncated at the C-terminal end compared with mammalian \( \gamma \)-carboxylase, which is 758 amino acids. The mammalian gene has 14 introns; in Drosophila there are two much shorter introns but in positions precisely homologous to two of the mammalian introns. In addition, a deletion of 6 nucleotides is observed when cDNA and genomic sequences are compared. In situ hybridization to fixed embryos indicated ubiquitous presence of carboxylase mRNA throughout embryogenesis. Northern blot analysis revealed increased mRNA levels in 12-24-h embryos. The continued presence of carboxylase mRNA suggests that it plays an important role during embryogenesis. Although the model substrate FLEEL is carboxylated by the enzyme, a substrate containing the propeptide of a Conus carboxylase substrate, conantokin G, is poorly carboxylated. Its occurrence in vertebrates, molluscan systems (i.e. Conus), and Drosophila and the apparently strong homology between the three systems suggest that this is a highly conserved and widely distributed post-translational modification in biological systems.

The functions of proteins are coordinated physiologically by post-translational modification. For example, phosphorylation-dephosphorylation cascades integrate the biochemistry of individual proteins into cellular physiology. In addition to post-translational modifications that occur primarily within cells, post-translational modifications also occur on extracellular proteins. The most familiar of these are \( N \)-glycosylation of asparagine residues and \( O \)-glycosylation of serine and threonine residues.

One of the most distinctive of the extracellular post-translational modifications is the vitamin K-dependent \( \gamma \)-carboxylation of glutamate residues to give \( \gamma \)-carboxyglutamate (1). When it was first characterized, \( \gamma \)-carboxylation was thought to be a biochemical specialization of the mammalian blood-clotting cascade. However, several bone proteins (2, 3) as well as an extracellular ligand, gas\( \delta \) (4), were subsequently identified as having the post-translational modification, although in the latter cases the precise mechanistic role of \( \gamma \)-carboxylation for proper protein function has not been established definitively. In addition, two novel proline-rich \( \gamma \)-carboxyglutamic acid-containing proteins, PRGP1 and PRGP2, of unknown function have been identified (5).

Long after its characterization in blood-clotting factors, vitamin K-dependent \( \gamma \)-carboxylation of glutamate residues was discovered in a phylogenetically distant system: the neuropeptides made in the venom duct of the predatory cone snails Conus (6, 7). The venoms of these snails have \( \sim 100 \) different peptides; \( \sim 5\% \) of these are believed to be \( \gamma \)-carboxylated (8). This post-translational modification has been found in a number of diverse Conus peptides but has been studied most intensively in an unusual Conus neuropeptide family, the conantokins, which are NMDA receptor antagonists.

In the conantokins, the significance of the post-translational modification can readily be demonstrated: these peptides are inactive in analogs without \( \gamma \)-carboxylation of glutamate residues. Incomplete \( \gamma \)-carboxylation of blood-clotting factors results in poor coagulation. It has been postulated that \( \gamma \)-carboxylation of both the conantokins and of factors of the blood-clotting cascade induces a helical conformation in the post-translationally modified regions. This postulated role of \( \gamma \)-carboxylation in determining conantokin structure has been generally supported by a number of subsequent structural studies on various conantokin (9–12). \( \gamma \)-Carboxyglutamic acid confers the property of \( Ca^{2+} \) binding to the modified protein. In the case of the blood-clotting factors, the binding to \( Ca^{2+} \) results in a conformational change exposing hydrophobic residues for interaction with membranes (13–17).

The enzymatic reaction in the invertebrate system has recently been shown to have many striking similarities (e.g. a requirement for reduced vitamin K and the presence of a \( \gamma \)-carboxylation recognition site on the substrate) to that of the \( \gamma \)-carboxylation of factors involved in the mammalian blood-clotting cascade (18, 19). Despite the clear functional importance of \( \gamma \)-carboxylation in these two disparate phylogenetic systems, \( \gamma \)-carboxylation of glutamate residues has been re-
garded as a highly specialized post-translational modification. In this report, we provide evidence that is strongly consistent with vitamin K-dependent γ-carboxylation in fact being a much more widely distributed biological phenomenon. We demonstrate by molecular techniques the presence of a vitamin K-dependent γ-carboxylase-related protein that is expressed in the fruit fly Drosophila melanogaster, which has a high degree of sequence identity with the mammalian enzyme. Similar observations have recently been reported by Li et al. (20). Although the role of γ-carboxylation in Drosophila remains unknown, this post-translational modification is present in arthropods, suggesting that it is generally distributed in animal systems. The strong conservation in sequence of the γ-glutamyl carboxylase in Drosophila and in mammals suggests an important functional role for the enzyme, resulting in strong selection for sequence conservation.

EXPERIMENTAL PROCEDURES

Materials—Conus textile venom ducts were obtained from Dr. L. J. Cruz (University of the Philippines). Vitamin K (phynobione) was from Abbott Laboratories, and NaH14CO3 (55 mCi/mmol) was from PerkinElmer Life Sciences. Enzymes were purchased from Life Technologies, Inc. PCR reactions were performed in an Air Thermocycler (Idaho Technology). Oligonucleotides were synthesized at the peptide sequencing facility at the University of Utah.

Preparation of mRNA—Adult Drosophila (Oregon) were frozen in liquid nitrogen and then ground to a fine powder, and total RNA was isolated (21). Poly(A)+ RNA was isolated using a Qiagen Oligotex mRNA kit according to the vendor’s instructions. Molecular biology experiments were done according to methods described by Sambrook et al. (22).

Sequence Analysis—The cDNA sequence of Drosophila γ-carboxylase was assembled from sequences of PCR products obtained by amplification of oligo(dT) or Q primer DNA using primers shown in Table I. The primer combinations used in the PCR are shown in Table I. Primers 1 and 2 correspond to amino acid sequence conserved in human, bovine, and rat. Primers 5 and 6 were selected from the Drosophila genomic sequence (Berkeley Drosophila Genome Project, accession number AC005557). 3′-Sequences of the carboxylase mRNAs were determined by the technique of rapid amplification of cDNA ends determined by the technique of rapid amplification of cDNA ends described by Frohman (23). The PCR product was cloned into the TA vector Bluecript (Life Technologies). 24 h after transfection cells were induced with 0.7 mM CuSO4, 48 h after induction 50% of the cells expressed GFP as judged by fluorescent microscopy. The results also indicated that the introns were properly processed, and a continuous reading frame was present in the cloned GC. rPmA-3.GC.GFP was modified to introduce a stop codon at the end of the GC coding sequences, and two internal GFP coding sequences. The modified plasmid rPmA-3.GC* was transferred into S2 cells. The cells were induced with 0.7 mM CuSO4 and harvested 48 h after induction. Cells were washed twice with phosphate-buffered saline and resuspended in buffer containing 25 mM 4-morpholinepropanesulfonic acid, pH 7.0, 0.5 mM NaCl, 0.2% 3-[3-cholamidopropyl]dimethylammonio]-1-propanesulfonic acid/phosphatidyl choline, 2 mM EDTA, 2 mM dithiothreitol, 0.2 μM/ml leupeptin, 0.8 μg/ml pepstatin, and 0.04 mM/ml phenylmethyisulfonyl fluoride. The cell suspension was briefly sonicated using a Branson 450 sonifer and incubated in ice for 20 min. The lysate was assayed for carboxylase activity.

Enzyme Assays—γ-Glutamyl carboxylase assays were performed as described by Stanley et al. (18). Reactions were done in a total volume of 125 μl containing cell lysate and a final concentration of reagents as follows: 25 mM 4-morpholinepropanesulfonic acid, pH 7.4, 0.5 mM NaCl, 0.2% 3-[3-cholamidopropyl]dimethylammonio]-1-propanesulfonic acid/phosphatidyl choline, 2 mM EDTA, 2 mM dithiothreitol, 0.2 μM/ml leupeptin, 0.8 μg/ml pepstatin, and 0.04 mM/ml phenylme thylsulfonyl fluoride. The cell suspension was briefly sonicated using a Branson 450 sonifer and incubated in ice for 20 min. The lysate was assayed for carboxylase activity.

RESULTS

Characterization of a Putative Drosophila γ-Carboxylase mRNA Sequence—Poly(A)+ RNA from adult Drosophila was used as template for the reverse transcription of cDNA using an oligo(dT) primer. An initial segment of cDNA was amplified and sequenced using γ-carboxylase primers encoding amino acid sequences highly conserved between Drosophila and mammalian enzymes; the 5′-oligonucleotide primer (primer 1; Table I) corresponded to amino acids 395–402, YGYSWDDMM, and the 3′-primer (primer 2; Table I) corresponded to amino acids 465–471, IYFDIWC (the amino acid positions correspond to human γ-glutamyl carboxylase sequence). The PCR product was cloned, and the nucleic acid sequence was determined; the
sequence obtained was identical to a DNA sequence in the Drosophila genome (BDGP, accession number AC005557).

We carried out further PCR analysis of the cDNA using the primers indicated schematically in Table I and Fig. 2A. The 3'-end of the transcript to the poly(A) addition site was determined using 3'-rapid amplification of cDNA ends. Polyadenylation takes place 20 bases downstream of a consensus Poly(A) signal, AATAAAA. The remainder of the cDNA was characterized by PCR amplification using primers shown in Table I; PCR products were cloned, and overlapping sequences were combined to yield the cDNA shown schematically in Fig. 2A.

The cDNA sequence encodes an open reading frame of 670 amino acids. Examination of the genomic sequence revealed two notable differences from the cDNA. First, there are two short introns. A schematic comparison of the genomic and cDNA, shown in Fig. 2B, illustrates the position of these introns. The nucleic acid sequences at the splice junction are shown in Fig. 2C. Second, an as yet uncharacterized processing event removes 6 nucleotides from the cDNA (Fig. 2D).

The locations of the two introns are conserved between Drosophila and mammals. As is generally found when comparing Drosophila with mammalian introns (30), the Drosophila introns are significantly shorter (for intron 1, 58 versus 2204 nucleotides; for intron II, 72 versus 646 nucleotides). Comparison of the amino acid sequences flanking Drosophila intron II and human intron VII is shown in Fig. 2E. There are a total of 14 introns in both the rat (26) and the human γ-carboxylase genes (31); therefore, Drosophila has both fewer and shorter introns compared with the mammalian gene. Fig. 3A shows a schematic of amino acid homology between human and Drosophila γ-carboxylase.

The surprising finding from the sequencing described above is that an uncharacterized mechanism of RNA processing resulted in the deletion of 6 nucleotides that would have been present if the DNA were faithfully transcribed. The deletion does not change the amino acid homology to the mammalian open reading frame.

With regard to the protein length, the alignment in Fig. 3B shows that although the Drosophila enzyme has 17 additional amino acids at the N terminus compared with the mammalian enzymes, it is significantly shorter at the C-terminal end. All of the mammalian enzymes are longer (758 versus 670 amino acids). A recent deletion analysis (32) of the bovine enzyme suggests that small deletions at the C terminus may be tolerated by the wild-type mammalian enzyme. Interestingly, a deletion that resulted in a bovine enzyme that was 676 amino acids in length had lower enzymatic activity (15-fold lower with respect to γ-carboxylation and 400-fold lower than for vitamin K epoxidation). It remains to be determined whether other Drosophila subunits are necessary to compensate for the shorter length of the Drosophila open reading frame.

Northern Blot Analysis—Fig. 4A shows the results of Northern blot analysis. Drosophila γ-glutamyl carboxylase mRNA is ~2.7 kb in size (Fig. 4A) and is predominantly expressed in 12-24-h embryos (Fig. 4B). (However, the more sensitive in situ hybridization experiments presented below reveal the presence of carboxylase mRNA throughout embryogenesis.) Ribosomal protein rp49 mRNA was also monitored in these experiments.
probes cross-hybridized to embryonic mRNAs in situ (Fig. 5, B and D). No hybridization-positive embryos were observed when sense transcripts were used as probes (Fig. 5, A and C).

**Assays for γ-Carboxylation Activity**—Lysates of S2 cells transfected with DNA encoding the putative γ-glutamyl carboxylase were assayed for carboxylation activity. Table II demonstrates that S2 cells transfected with pRmHa-3.GC* expresses vitamin K-dependent γ-carboxylase activity that is not present in mock-transfected cells or cells transfected with pRmHa-3.GFP (data not shown).

**Substrate Specificity of γ-Carboxylation**—Endogenous substrates of both the mammalian (35) and Conus γ-glutamyl carboxylases contain a γ-carboxylation recognition signal (γ-CRS) in the propeptide region (19, 36). The presence of a γ-CRS at the amino terminus of a substrate greatly enhances the efficiency of carboxylation. We have previously identified the γ-CRS of a γ-carboxylated Conus peptide, conantokin-G (19). A peptide containing proconantokin-G sequences (−20 to +5, 2GKDRLTQMRKILQRGKNKAR−GEEEL+5Y) referred to as −20Y in the text below, is efficiently carboxylated by Conus carboxylase (19). We determined the carboxylation of both FLEEL and −20Y by Conus and Drosophila γ-glutamyl carboxylases. The ratio of 14CO2 incorporated in the two substrates (−20Y/FLEEL) by the Conus enzyme was 4, whereas that for the Drosophila enzyme was 0.26.

**DISCUSSION**

In this work, we have characterized a cDNA clone derived from adult *D. melanogaster* mRNA that encodes a protein with γ-glutamyl carboxylase activity. The open reading frame is homologous to the mammalian γ-glutamyl carboxylase throughout its length of 670 amino acids; the degree of sequence identity is strikingly high (39% identity and 55% homology) and comparable with the subset of enzymes highly conserved between *Drosophila* and mammals. One unexpected result when comparing the putative genomic DNA sequence with the cDNA is that there is an apparent small deletion in the cDNA sequence, suggesting that RNA processing of the *Drosophila* γ-glutamyl carboxylase mRNA occurs.

Northern blot analysis indicates that the putative *Drosophila* γ-glutamyl carboxylase mRNA is ~2.7 kb in size. This is ~350 nucleotides longer than the open reading frame and 3′-untranslated region previously characterized from cDNA. Because a single RNA isoform is observed, and the 3′-end of the RNA determined by cDNA analysis is unique, the additional 350 nucleotides represent the length of the poly(A) tail together with the 5′-untranslated region. In situ hybridization reveals the ubiquitous presence of carboxylase RNA throughout embryogenesis. By reverse transcription-PCR analysis, Li et al. (20) have also reported similar observations. In early embryos (0–2 h) it is probably of maternal origin, whereas in later embryos (2–12 h), there is considerable contribution from embryonic synthesis as suggested by the kinetics of RNA synthesis. In this regard, carboxylase mRNA expression has been demonstrated in neuronal and skeletal tissue of postimplantation rat embryos during early organogenesis (26).

The discovery that a γ-carboxylase is expressed in *Drosophila* opens the door to genetic analysis of vitamin K-dependent γ-carboxylation in this model system. The availability of the *Drosophila* γ-glutamyl carboxylase sequence already provides significant structure-function information. Some segments are clearly much more highly conserved when the *Drosophila* sequence is compared with that of mammalian enzymes; presumably, these are domains of the enzyme most critical for function. Among the most noteworthy, mammalian and *Drosophila* sequences corresponding to amino acids 385–404 in the *Drosophila* sequence (see Fig. 3) are identical. An extended con-
Heterologous γ-CRS sequences are not or are poorly recognized by the γ-glutamyl carboxylases. γ-CRS containing Conus substrate, proconantokin G, is poorly carboxylated by the bovine enzyme, whereas a peptide, factor IXA–41, which consists of the propeptide and all normally carboxylated residues of the vitamin K-dependent clotting protein factor IX, is not carboxylated by the Conus enzyme (18). The poor carboxylation of γ-CRS by the Drosophila enzyme further strengthens the suggestion that the enzymes have evolved to recognize their cognate γ-CRSs. This is also supported by the observation of Li et al. (20), who found that the propeptide of human blood coagulation factor IX did not stimulate carboxylation by the Drosophila enzyme. Because the Drosophila and human γ-glutamyl carboxylases share considerable sequence homology, it should be possible to identify substrate binding domains by studying carboxylation using chimeric enzymes.

Because γ-carboxylated molecules may serve as signals for growth and differentiation, differential regulation of γ-carboxylation may operate at multiple levels during development. Control may be at the level of synthesis of γ-carboxylase mRNA or its translation, or both. Although mRNA may be present, enzyme activity may not be obvious. Future experiments will be aimed at determining possible differences among levels of mRNA, expressed protein, and activity by immunological methods (for protein) and γ-carboxylase assay (for activity).

γ-Carboxyglutamate-containing proteins isolated to date are extracellular proteins. γ-Carboxyglutamate interacts with Ca²⁺, induces a conformational change in the protein, and facilitates binding to membrane phospholipids. A number of γ-carboxyglutamate-containing vitamin K-dependent proteins (thrombin, factor Xa, protein S, and Gas6) are ligands for cell surface receptors. Interaction with the receptors induces cellular proliferative responses (39, 40). In Drosophila, high levels of γ-carboxylase RNA are detected in late stage embryos. During this period, a variety of developmental and morphogenetic events occur, among them cuticle deposition and central nervous system, peripheral nervous system, and gut differentiation. It is conceivable that some of the gene products signaling these events are γ-carboxylated and serve as ligands for corresponding receptors. The effects of γ-carboxylase knockout in flies will enable a systematic study of probable targets for this post-translational modification.

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REFERENCES

1. Stenflo, J., Fernlund, P., Egan, W., and Roeserhoff, P. (1974) Proc. Natl. Acad. Sci. U. S. A. 71, 2730–2733
2. Price, P. A., and Williamson, M. K. (1985) J. Biol. Chem. 260, 14971–14975
3. Pan, L. C., and Price, P. A. (1985) Proc. Natl. Acad. Sci. U. S. A. 82, 6159–6163
4. Maniotti, G., Bracewell, C., Avanzini, G., and Schneider, C. (1993) Mol. Cell. Biol. 13, 4976–4985
5. Kalman, J. D., Harris, J. E., Haldeman, B. A., and Dave, E. W. (1997) Proc.
γ-Glutamyl Carboxylase in Drosophila

6. McIntosh, J. M., Olivera, B. M., Cruz, L. J., and Gray, W. R. (1984) J. Biol. Chem. 259, 14343–14346
7. Craig, A. G., Bandyopadhyay, P., and Olivera, B. M. (1999) Eur. J. Biochem. 264, 271–275
8. Haushka, P. V., Mullen, E. A., Hintsch, G., and Jazwinski, S. (1988) in Current Advances in Vitamin K Research (Suttie, J. W., ed) pp. 237–243, Science Publishers, New York
9. Blandl, T., Zajicek, J., Prorok, M., and Castellino, F. J. (1997) Biochem. J. 328, 777–783
10. Rigby, A. C., Baleja, J. D., Furie, B. C., and Furie, B. (1997) Biochemistry 36, 6906–6914
11. Warder, S. E., Chen, Z., Zhu, Y., Prorok, M., Castellino, F. J., and Ni, F. (1997) FEBS Lett. 411, 19–26
12. Skjaebaek, N., Nielsen, K. J., Lewis, R. J., Alewood, P., and Craik, D. J. (1997) J. Biol. Chem. 272, 2291–2299
13. Myers, R. A., McIntosh, J. M., Imperial, J., Williams, R. W., Oas, T., Haack, J. A., Hernandez, J.-F., Rivier, J., Cruz, L. J., and Olivera, B. M. (1990) J. Toxicol. Toxicol. Rev. 9, 179–202
14. Soriano-Garcia, M., Padmanabhan, K., de Vos, A. M., and Tulinsky, A. (1992) Biochemistry 31, 2554–2566
15. Freedman, S. J., Furie, B. C., Furie, B., and Baleja, J. (1995) J. Biol. Chem. 273, 5447–5450
16. Sunnerhagen, M., Forsen, S., Hoffren, A. M., Drakenberg, T., Teleman, O., and Stenflo, J. (1995) Nat. Struct. Biol. 2, 504–509
17. Mann, K. G., Nesheim, M. E., Church, W. R., Haley, P., and Krishnawamy, S. (1990) Blood 76, 1–16
18. Stanley, T. B., Stafford, D. W., Olivera, B. M., and Bandyopadhyay, P. K. (1997) FEBS Lett. 407, 85–88
19. Bandyopadhyay, P. K., Collardeau, C. J., Walker, C. S., Zhou, L.-M., Hillyard, D. R., and Olivera, B. M. (1998) J. Biol. Chem. 273, 5447–5450
20. Li, T., Yang, C.-T., Jin, D., and Stafford, D. W. (2000) J. Biol. Chem. 275, 18291–18296
21. Chomczynski, P., and Sacchi, N. (1987) Anal. Biochem. 162, 156–159
22. Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989) Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Press, Cold Spring Harbor, NY
23. Frohman, M. A. (1994) in The Polymerase Chain Reaction (Muliss, K. B., Ferré, F., and Gibbs, R. A., eds) pp. 14–37, Birhauser, Boston
24. Wu, S.-M., Cheung, W.-F., Frazier, D., and Stafford, D. W. (1991) Science 254, 1634–1636
25. Rehemtulla, A., Roth, D. A., Wasley, L. C., Kulipolus, A., Walsh, C. T., Furie, B., Furie, B. C., and Kaufman, R. J. (1993) Proc. Natl. Acad. Sci. U. S. A. 90, 4611–4615
26. Romero, E. E., Velazquez-Estades, L. J., Dee, R., Schapiro, B., and Roth, D. A. (1989) Exp. Cell. Res. 243, 334–346
27. Lehmann, R., and Tautz, D. (1994) in Methods in Cell Biology. (Goldstein, L. S. B., and Gyrberg, E. A., eds) Vol. 44, pp. 575–588, Academic Press, New York
28. Bunch, T. A., Grinblat, Y., and Goldstein, L. S. B. (1988) Nucleic Acids Res. 16, 1043–1061
29. Esmon, C. T., Sadowski, J. A., and Suttie, J. W. (1975) J. Biol. Chem. 250, 4744–4748
30. Deutsch, M., and Long, M. (1999) Nucleic Acids Res. 27, 3219–3228
31. Stafford, D. W., Frazier, L. D., Fu, Y. Y., High, K. A., Chu, K., Sanchez-Vega, B., and Solera, J. (1997) Blood 88, 4058–4062
32. Roth, D. A., Whirl, M. L., Velazquez-Estades, L. J., Walsh, C. T., Furie, B., and Furie, B. C. (1995) J. Biol. Chem. 270, 5305–5311
33. Andres, A. J., and Cherbas, P. (1992) Development 116, 865–876
34. Stronach, B. E., Siegrist, S. E., and Beckerle, M. C. (1996) J. Cell Biol. 134, 1179–1195
35. Kulipolus, A., Cieruzzo, C. E., Furie, B., Furie, B. C., and Walsh, C. T. (1992) Biochemistry 31, 9436–9444
36. Bush, K. A., Stenflo, J., Roth, D. A., Czerwiec, E., Harrist, A., Begley, G. S., Furie, B. C., and Furie, B. (1999) Biochemistry 38, 14660–14666
37. Begley, G. S., Furie, B. C., Czerwiec, E., Taylor, K. L., Furie, G. L., Bronstein, L., Stenflo, J., and Furie, B. (2000) J. Biol. Chem., 275, 36245–36249
38. Brenner, B., Sanchez-Vega, B., Wu, S., Lainir, N., Stafford, D. W., and Solera, J. (1998) Blood 92, 4554–4559
39. Esmon, C. T. (1995) Curr. Biol. 5, 743–746
40. Crosier, K. E., and Crosier, P. S. (1997) Pathology 29, 131–135