Gene duplications in the prototypical cephalochordate amphioxus

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Abstract
The new discipline of Evolutionary Developmental Biology (Evo-Devo) is facing the fascinating paradox of explaining morphological evolution using conserved pieces or genes to build divergent animals. The cephalochordate amphioxus is the closest living relative to the vertebrates, with a simple, chordate body plan, and a genome directly descended from the ancestor prior to the genome-wide duplications that occurred close to the origin of vertebrates. Amphioxus morphology may have remained relatively invariant since the divergence from the vertebrate lineage, but the amphioxus genome has not escaped evolution. We report the isolation of a second Emx gene (AmphiEmxB) arising from an independent duplication in the amphioxus genome. We also argue that a tandem duplication probably occurred in the Posterior part of the Hox cluster in amphioxus, giving rise to AmphiHox14, and discuss the structure of the chordate and vertebrate ancestral clusters. Also, a tandem duplication of Evx in the amphioxus lineage produced a prototypical Evx gene (AmphiEvxA) and a divergent gene (AmphiEvxB), no longer involved in typical Evx functions. These examples of specific gene duplications in amphioxus, and other previously reported duplications summarized here, emphasize the fact that amphioxus is not the ancestor of the vertebrates but ‘only’ the closest living relative to the ancestor, with a mix of prototypical and amphioxus-specific features in its genome. © 2002 Elsevier Science B.V. All rights reserved.

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1. Introduction

Evolutionary biology and developmental biology are finally being married after nearly 150 years of stormy courtship. After an initial cosy relationship in the late 19th and early 20th centuries, they went their separate ways, due to differences of opinion and interests. In the late 20th century the impact of molecular biology has facilitated a reconciliation. The result of this reconciliation has been the rejuvenation of the discipline of Evolutionary Developmental Biology, or Evo-Devo (Raff, 2000). Evo-Devo is concerned with how developmental processes themselves change and evolve. The rationale being that evolution is change in morphology, morphology depends on embryonic development, and development depends on developmental genes, gene networks, and modules. Thus, evolution can be seen as the result of adaptive variation in development (Von Dassow and Munro, 1999). Experimental work in Evo-Devo has produced a major generalization: major genes, gene networks and modules are conserved amongst most animal phyla. This universality of developmental genes has lead to the exciting paradox of Evo-Devo: how can one evolve with conserved genes and modules, if evolution is basically change? Subtle changes in cis-regulatory regions and in hierarchies and connections within and between networks may give clues to the paradox (see Salazar-Ciudad et al., 2001 for a theoretical view). A further mechanism of genetic change that can enable evolution without necessarily compromising previous developmental functions is gene duplication.

2. Gene duplication, amphioxus and the origin of vertebrates

The late Susumo Ohno’s heavily cited book, Evolution By
Gene Duplication (Ohno, 1970) was a visionary anticipation of the importance of gene duplication in evolution. After duplication, one of the gene copies may be less constrained, allowing innovation and diversification. Ohno also proposed that two rounds of full genome duplication occurred at the origin of vertebrates.

Lundin reported widespread paralogy regions in the human genome, and interpreted them as remnants of duplications of large genomic regions, or ancient polyploidy (Lundin, 1993). This, together with the discovery that cephalochordates, the sister group of the vertebrates, had a single Hox cluster, prototypical to the four Hox clusters of mammals (Garcia-Fernández and Holland, 1994), strengthened the hypothesis that two full polyploidizations occurred at the origin of vertebrates (Holland et al., 1994). These genome duplications intriguingly correlate with the origin of vertebrates and their many innovations.

The nature and extent of these duplications have been debated in recent years, but it is now beyond doubt that widespread duplications did occur in the vertebrate lineage, most probably via polyploidizations. The precise locations and extent of these events within a vertebrate phylogeny is now becoming the focus of the debate (Holland, 1999). Amphioxus thus occupies a key phylogenetic position, being the sister group to the vertebrates, but moreover being the lineage closest to these genome-wide duplications before they occurred. Morphologically amphioxus is considered to be not excessively derived from the cephalochordate-vertebrate common ancestor. Cephalochordates possess a hollow dorsal neural tube, a notochord, and lateral muscle blocks, as is typical for chordates (urochordates, cephalochordates and vertebrates), but lack the vertebrate innovations. The origin of vertebrates involved the origin of new cell types and organs, and increased body organization complexity. Vertebrate innovations include neural crest cells, neurogenic placodes, an elaborated and morphologically segmented brain, paired sense organs and endoskeleton (Shimeld and Holland, 2000).

The phylogenetic position of amphioxus as the sister group of vertebrates, and the simple and prototypical body plan of amphioxus compared to vertebrates, has led many to examine their favourite gene (or gene family) in this animal in order to ascertain the ancestral state for vertebrates. This has proven to be a fruitful venture (see Holland, 1999 for a catalogue), but it must always be remembered that amphioxus itself has been evolving from the common ancestor of cephalochordates and vertebrates for over 550 Myr. We will present three examples of the amphioxus genome reflecting both its closeness to the pre-duplication vertebrate ancestor, but also its derived condition. These amphioxus novelties are gene duplications specific to the amphioxus lineage.

3. Posterior Hox genes: a chordate Hox14

Hox genes pattern the anterior–posterior axis of animals and are organized into clusters in the genome. Within a cluster the genes are organized such that they are colinear with their domains of expression and function: the Anterior patterning genes are at one end of the cluster (the 3’ end), the Medial genes are in the centre of the cluster, and the Posterior-patterning genes at the other end (5’). This phenomenon of colinearity is seen from flies to humans, including amphioxus (Graham et al., 1989; Wada et al., 1999). The multiple Hox clusters of vertebrates, e.g. four...
in mammals and seven in zebrafish (Amores et al., 1998), contain 13 different types of Hox gene (paralogy groups), divisible into the Anterior, Group 3, Medial and Posterior classes (Fig. 1). The Posterior class consists of Hox groups 9–13. Amphioxus, consistent with its lineage diverging from that of vertebrates before the genome-wide duplications at vertebrate origins, has a single Hox gene cluster. In many respects the amphioxus cluster can be considered to be prototypical relative to those of vertebrates. It has no gaps, and contains a pro-orthologue for each of the vertebrate paralogy groups, at least up to the Posterior region of the cluster (Garcia-Fernández and Holland, 1994). In the Posterior region the situation is not so straightforward. Amphioxus may not have genes missing, but it does contain an extra gene (Hox 14), and the distinctive evolutionary rates of these Posterior genes reveal the phenomenon of Deuterostome Posterior Flexibility, whereby the rate of evolution of the deuterostome Posterior Hox genes has increased relative to the more anterior Hox genes, and the protostome Posterior Hox genes (Ferrier et al., 2000). This obscures the relationships between the deuterostome Posterior Hox genes.

The 14th amphioxus Hox gene was found during a genomic walk from the Posterior/S5′ end of the Hox cluster. Its discovery gives amphioxus the (dubious) honour of possessing the most gene-rich Hox cluster to date. Did AmphiHox14 arise from a tandem duplication only within the amphioxus lineage, or does it indicate that the vertebrate ancestor also had a fourteenth Hox gene, which has subsequently been lost in at least some vertebrates (only humans, mice, and zebrafish have been examined in sufficient detail to unambiguously determine the absence of a Hox14)? Molecular phylogenetics has not clearly resolved this issue (Ferrier et al., 2000). There is a faint suggestion from some phylogenetic trees that AmphiHox13 and 14 form a sister group, which is consistent with the fourteenth Hox gene being an amphioxus-specific feature. This grouping however is not stable amongst all trees, or with various different molecular phylogenetic approaches. Furthermore since AmphiHox13 and 14 are two of the most divergent sequences in these trees there is the possibility that their grouping is artefactual, due to their longer branches attracting each other.

As for the vertebrate ancestor, we favour the hypothesis that it contained 13 Hox genes orthologous to AmphiHox 1–13, with AmphiHox14 being an amphioxus-specific duplication, and the orthology relationships between the amphioxus and vertebrate genes being obscured by Deuterostome Posterior Flexibility. The possibility that it contained a Hox14 gene, however, cannot yet be completely excluded.

Resolution of the ancestral state would only conclusively come with the discovery of a fourteenth vertebrate Hox gene. However it would also be intriguing to know precisely how many Posterior genes other deuterostome taxa have. This would require a thorough genomic walk from the end of the Hox cluster in the respective animal. The divergent nature of the Posterior-most Hox genes means that degenerate PCR approaches alone are not sufficient. In the sea urchin, genomic walking has been done as far as three Posterior Hox genes (Martinez et al., 1999). There are probably more further 5′, as up to five Posterior Hox genes have been cloned (by PCR) from other species of echinoderm (Méndez et al., 2000; Mito and Endo, 2000). The sea urchin genome project will tidy this up. However, it must also be borne in mind that this echinoderm also exhibits an example of Hox gene loss. It lacks either a Hox 4 or 5 gene (Martinez et al., 1999). Another animal in which a genome project might help is the urochordate Ciona intestinalis. Three Posterior Hox genes have so far been isolated in C. intestinalis (Di Gregorio et al., 1995), but considerable effort with genomic walking has so far been unsuccessful in linking all of the ascidian Hox genes together (Di Lauro and colleagues, personal communication). Of course, amphioxus may have still more Posterior Hox genes. So far the walk has been extended 80 kb beyond AmphiHox14, without uncovering any more Hox genes (C. Minguillón and J. Garcia-Fernández, unpublished). However, linkage to the Evx genes has not yet been established as a means to confirm that the end of the cluster has been reached (see Section 4).

4. Prototypical and divergent Evx genes after tandem gene duplication in the amphioxus lineage

Evx homeobox genes are present from cnidarians to vertebrates. They seem to have a basic function in patterning the posterior part of the embryo at the onset of gastrulation in all bilaterians, and also function in the central nervous system. Lineage-specific functions have also been acquired: even-skipped is a Pair-Rule gene in Drosophila melanogaster segmentation, whilst in vertebrates Evx genes function in development of the tail and limb buds, and are expressed around the Midbrain/Hindbrain Boundary (MHB) (Ferrier et al., 2001 and references therein). The genes are linked to the posterior ends of Hox clusters in vertebrates, and an Evx gene has been reported closely linked to a Hox-like gene in corals, a characteristic probably shared with other cnidarians (Ferrier and Holland, 2001). We isolated Evx representative(s) from amphioxus and traced the evolutionary history of Evx in chordates (Ferrier et al., 2001). We found two linked Evx genes in amphioxus (AmphiEvxA and AmphiEvxB), only 35 kb apart (schematized in Fig. 1). AmphiEvxA and AmphiEvxB probably arose via tandem gene duplication. The homeodomain of AmphiEvxA is clearly closer to vertebrate Evx1 and Evx2 than the homeodomain of AmphiEvxB, whose similarity to the vertebrate proteins is distinctly lower (Table 1). This suggests that AmphiEvxA is prototypical while AmphiEvxB is a divergent member of the family. Molecular phylogenetic analyses of the Evx genes confirmed that AmphiEvxA lies at the base of vertebrate genes, while AmphiEvxB is a divergent Evx. AmphiEvxA is expressed during three developmental
AmphiEvxA is expressed in the ventral-posterior domain (Fig. 2A–C). At neurula and early larval stages AmphiEvxA is expressed at the posterior of the embryo, in all three germ layers (data not shown), with clear expression in the central nerve cord. The pairs of cells in the CNS are seen at intervals equivalent to the length of one somite, but never more anterior than the level of the somite 4/5 boundary, with the pair at the level of the somite 5/6 boundary being most persistent (Fig. 2D). Finally, in larval stages AmphiEvxA is expressed in the tail bud, in the posterior-most neural tube and endoderm (Fig. 2E). AmphiEvxB expression is not detectable until hatching, when a consistent stain all over the ectoderm appears and is maintained until larval stages (Fig. 2F).

Both sequence comparisons and expression patterns clearly indicate that AmphiEvxA is a prototypical chordate Evx gene. The basal chordate role of Evx genes was the pan-bilaterian function in gastrulation and neurogenesis, plus a probable pan-chordate role in tail bud development. The expression of Evx around the MHB is probably a vertebrate innovation, perhaps linked to the evolution of the MHB.

### Table 1
Percentage of identities to AmphiEvxA and B homeodomains and six amino acid flanks

<table>
<thead>
<tr>
<th></th>
<th>% Identities to AmphiEvxA</th>
<th>% Identities to AmphiEvxB</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vertebrate Evx1</td>
<td>m Evx 1</td>
<td>86</td>
</tr>
<tr>
<td></td>
<td>X Xhox3</td>
<td>86</td>
</tr>
<tr>
<td></td>
<td>zf Evx 1</td>
<td>85</td>
</tr>
<tr>
<td>Vertebrate Evx2</td>
<td>m Evx 2</td>
<td>88</td>
</tr>
<tr>
<td></td>
<td>zf Evx 2</td>
<td>85</td>
</tr>
<tr>
<td>Invertebrate Eve</td>
<td>Dm Eve</td>
<td>82</td>
</tr>
<tr>
<td></td>
<td>Ce Vab7</td>
<td>69</td>
</tr>
<tr>
<td></td>
<td>coral EveC</td>
<td>82(^{b})</td>
</tr>
</tbody>
</table>

\(^{a}\) Species abbreviations: Ce, Caenorhabditis elegans; Dm, Drosophila melanogaster; m, mouse; X, Xenopus; zf, zebrafish.

\(^{b}\) No flanking regions of coral EveC are available. Percentages refer only to the homeodomain.
organizer activity in vertebrates. Appendages, and the function of Evx in their development, evolved within the vertebrates. In summary, an ancestral Evx gene was duplicated in the amphioxus genome, giving rise to a prototypical Evx gene (AmphiEvxA) and a fast evolving gene (AmphiEvxB), that is no longer involved in typical Evx functions.

5. Duplication in amphioxus of an Emx-class homeobox gene

Emx genes are expressed and function in anterior cephalic domains during embryonic development of vertebrates and D. melanogaster (Panesse et al., 1998 and references therein). Orthologues have also been found in Caenorhabditis elegans, the hydrozoan Hydractinia symbiolongicarpus, and recently in the cephalochordate amphioxus (AmphiEmxA, Williams and Holland, 2000).

Vertebrates possess two Emx genes (Emx1 and 2) arising from the genome-wide duplications at the origin of vertebrates. Emx genes were also independently duplicated in the lineage leading to higher insects, giving raise to empty-spiracles (ems) and E5 genes in D. melanogaster (Walldorf and Gehring, 1992).

Here we report the cloning of a second AmphiEmx gene, AmphiEmxB. The cDNA sequence has been deposited in GenBank under accession number AY040834.

**Fig. 3.** cDNA nucleotide sequence and predicted amino acid sequence of AmphiEmxB. The homeodomain residues are shown in bold, and the conserved Emx peptide domain near the N-terminus is underlined. Asterisks indicate the first 5' and 3' in-frame stop codons. Deduced intron positions are indicated with black triangles. The Emx cDNA sequence has been deposited in GenBank under accession number AY040834.

**Fig. 4.** Alignment of the homeodomain sequence of AmphiEmxA and B with vertebrate, fly, nematode and cnidarian homeodomain sequences. Dashes indicate identities to AmphiEmxB. The percentages of identity to the AmphiEmxB or AmphiEmxA homeodomains are shown. Species abbreviations are: Ce, Caenorhabditis elegans; Cn, cnidarian (Hydractinia symbiolongicarpus); Dm, Drosophila melanogaster; h, human; m, mouse; X, Xenopus; zf, zebrafish.
AmphiEmxA and B are 85% similar in the homeodomain, with only scattered similarities outside: two small domains can be recognized and aligned, the hexapeptide just upstream of the homeodomain, and the Emx peptide domain (Williams and Holland, 2000) at the amino-terminus of the protein, where they are 78.6% similar (underlined in Fig. 3).

A Neighbour-joining tree using 1000 bootstrap replicates, with AmphiEmxA and B and representative vertebrate Emx homeodomains (incomplete sequences have been omitted), and rooted with Drosophila Ems, shows that the amphioxus genes group together, and form a sister group to both vertebrate Emx1 and 2 classes (Fig. 5). This topology is consistent with vertebrate and amphioxus Emx genes having arisen by independent duplications in both lineages.

A closer look at the sequence similarities (Fig. 4) and branch lengths (Fig. 5) suggests that AmphiEmxB is slightly more related to vertebrate genes than AmphiEmxA, and that its rate of evolution is lower than that of AmphiEmxA. With no expression data available, it is difficult to speculate on which copy, if any, or both, have retained an ancestral role in patterning the most anterior part of the neural tube, a presumed ancestral function for this gene family in chordates. However, based on the above reasoning, we speculate that AmphiEmxB may be the one retaining ancestral roles.

6. Further gene duplications in the amphioxus lineage

Table 2 summarizes published data on amphioxus-specific gene duplications. A further report of an extra actin gene from B. belcheri expressed in the notochord (Suzuki and Satoh, 2000) is not included as it is not clear whether it originated from a duplication of a cytoplasmic or a muscle actin gene. Where there is information about the expression of duplicated genes, the behaviour of the duplicates is diverse. For brachyury, duplicates may have indistinguishable patterns of expression, or the expression reported may be the summation of both genes (Holland et al., 1995). For HNF-3 and muscle actin genes the differences of expression are most probably quantitative (Shimeld, 1997; Kusakabe et al., 1997). AmphiEvx A and B, however, have entirely unrelated patterns of expression. It is noteworthy that a single RDH gene has been found in B. lanceolatum, whereas two

![Fig. 5. Neighbour-joining phylogenetic tree with homeodomain sequences of amphioxus AmphiEmxB, AmphiEmxA, vertebrate Emx proteins, and Drosophila Ems. The numbers at the nodes are scores from 1000 bootstrap resamplings of the data. The abbreviations used are the same as in Fig. 4.](image)

Table 2
List of reported gene duplications specific to the amphioxus lineage

<table>
<thead>
<tr>
<th>Genes</th>
<th>Type</th>
<th>Species</th>
<th>Number</th>
<th>Expression</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Brachyury</td>
<td>TF</td>
<td>B. floridæ</td>
<td>2</td>
<td>Unknown</td>
<td>Holland et al., 1995</td>
</tr>
<tr>
<td>Myogenic bHLH</td>
<td>TF</td>
<td>B. floridæ</td>
<td>2</td>
<td>Unknown</td>
<td>Araki et al., 1996</td>
</tr>
<tr>
<td>HNF-3</td>
<td>TF</td>
<td>B. floridæ</td>
<td>2</td>
<td>Very similar*</td>
<td>Shimeld, 1997</td>
</tr>
<tr>
<td>Muscle actin</td>
<td>STR</td>
<td>B. floridæ</td>
<td>2</td>
<td>Very similar*</td>
<td>Kusakabe et al., 1997</td>
</tr>
<tr>
<td>Cholinesterase</td>
<td>ENZ</td>
<td>B. lanceolatum</td>
<td>2</td>
<td>Unknown</td>
<td>Sutherland et al., 1997</td>
</tr>
<tr>
<td>Tyr K (Ephs)</td>
<td>SIG</td>
<td>B. belcheri</td>
<td>2</td>
<td>Unknown</td>
<td>Suga et al., 1999</td>
</tr>
<tr>
<td>Tyr K (src)</td>
<td>SIG</td>
<td>B. belcheri</td>
<td>2</td>
<td>Unknown</td>
<td>Suga et al., 1999</td>
</tr>
<tr>
<td>Hox 13/14</td>
<td>TF</td>
<td>B. floridæ</td>
<td>2</td>
<td>Unknown</td>
<td>Ferrier et al., 2000</td>
</tr>
<tr>
<td>Calmodulin</td>
<td>SIG</td>
<td>B. lanceolatum</td>
<td>2</td>
<td>Unknown</td>
<td>Karabinos and Bhattacharya, 2000</td>
</tr>
<tr>
<td>Calmodulin-like</td>
<td>SIG</td>
<td>B. lanceolatum</td>
<td>2</td>
<td>Unknown</td>
<td>Karabinos and Bhattacharya, 2000</td>
</tr>
<tr>
<td>PTP (PTPR4)</td>
<td>SIG</td>
<td>B. belcheri</td>
<td>3</td>
<td>Unknown</td>
<td>Ono-Koyanagi et al., 2000</td>
</tr>
<tr>
<td>Evx</td>
<td>TF</td>
<td>B. floridæ</td>
<td>2</td>
<td>Distinct</td>
<td>Ferrier et al., 2001</td>
</tr>
<tr>
<td>RDH</td>
<td>ENZ</td>
<td>B. floridæ</td>
<td>2</td>
<td>Unknown</td>
<td>Dalfó et al., 2001</td>
</tr>
<tr>
<td>Emx</td>
<td>TF</td>
<td>B. floridæ</td>
<td>2</td>
<td>Unknown</td>
<td>Present work</td>
</tr>
</tbody>
</table>

*a* bHLH, basic helix–loop–helix; Tyr K, tyrosine kinase; PTP, protein tyrosine phosphatases; RDH, retinol dehydrogenase; TF, transcription factor; STR, structural molecule; SIG, signalling molecule; ENZ, enzyme.

*b* Type of molecule encoded.

*c* Number of genes for the given family reported in amphioxus. In all cases, a single gene is expected in the last common ancestor of cephalochordates and vertebrates.

*d* When expression data are available, the relation between the duplicates is indicated. *Quantitative differences.*
are present in *B. floridæ* (Dalfø et al., 2001). The authors convincingly demonstrate that there is only a single copy in *B. lanceolatûm* and that the two copies of *B. floridæ* arose after an independent duplication. Taking into account that both species may have diverged as long as 190 Myr ago (Cañestro et al., 2002), it is perhaps not surprising to find species-specific gene duplications within these anciently separated *Branchiostóma* lineages.

7. Discussion

The rate of gene duplication and subsequent gene loss has been a matter of discussion for many years (see Meyer and Schartl, 1999 and references therein), and can now be addressed with the aid of complete genome sequences (e.g. Wolfe, 2001 and references therein). It has been proposed that gene duplication may occur at a high rate, over 0.01 per gene per million years, with gene loss after duplication being more common than maintaining a duplicate by functional divergence (Lynch and Conery, 2000). The origin of vertebrates may be an exception to the high rate of gene loss after gene duplication. It is clear that numerous gene families expanded by gene duplication on the vertebrate stem lineage. Although it is difficult to demonstrate experimentally, it is tempting to speculate that vertebrate innovations were possible due to the availability of extra genetic raw material after polyploidization or widespread gene duplications.

*Amphioxus* is in the privileged position of being morphologically not very derived, since the divergence from the vertebrate ancestor. The invariance of amphioxus morphology is realized by consideration of the estimates of sequence divergence between three amphioxus species: *B. floridæ* (West Atlantic Ocean), *B. lanceolatûm* (East Atlantic and Mediterranean), and *B. belcheri* (Pacific Ocean). The estimates range from 110 to 190 Myr (Nohara et al., 2001; Cañestro et al., 2002), for species of the same genus, estimates that are much higher than the divergence between primate and rodent genes (different orders). This implies that the morphological uniformity among living lancelets is attributable to morphological stability rather than to recent speciation, and by extension, amphioxus morphology has not evolved much from the cephalochordate-vertebrate ancestor.

However, the amphioxus genome has been evolving separately from that of vertebrates for over 550 Myr, and is thus a remnant of the ancestor’s plus the particular history of the amphioxus lineage, with gene duplications, functional divergence and gene losses. We have presented three examples of duplications: the probable expansion at the posterior end of the amphioxus Hox cluster, the tandem duplication of Evx, and duplication of an Emx class gene.

Amphioxus morphology may have remained astonishingly invariant since the origin of vertebrates, but the amphioxus genome has not escaped evolution. Gene duplication events are not so rare, as perhaps should be expected in any lineage with a long independent evolutionary history. Amphioxus has undergone duplication of developmentally important genes (Emx). Such duplications raise questions about the chordate and vertebrate ancestors; for example, Hox14 and how many Posterior Hox genes were there? Also duplication of genes can lead to very divergent members of their respective classes (*AmphiEvxB*), which may have unusual, atypical expression for the class, if indeed they are not recently formed pseudogenes. Gene duplications in amphioxus are not restricted to homeobox genes (see Table 2), and so when analysing an amphioxus gene to trace the history of a given gene family in chordates, special attention should be paid to the fact that amphioxus is not the ancestor of vertebrates, but ‘only’ the closest living relative to the vertebrate ancestor, an inviable position to help understand the origin of vertebrates.

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