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Identification of Two *Drosophila* TGF-β Family Members in the Grasshopper *Schistocerca americana*

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Abstract. Intercellular signaling molecules of the transforming growth factor-β (TGF-β) superfamily are required for pattern formation in many multicellular organisms. The *decapentaplegic* (*dpp*) gene of *Drosophila melanogaster* has several developmental roles. To improve our understanding of the evolutionary diversification of this large family we identified *dpp* in the grasshopper *Schistocerca americana*. *S. americana* diverged from *D. melanogaster* approximately 350 million years ago, utilizes a distinct developmental program, and has a 60-fold-larger genome than *D. melanogaster*. Our analyses indicate a single *dpp* locus in *D. melanogaster* and *S. americana*, suggesting that *dpp* copy number does not correlate with increasing genome size. Another TGF-β superfamily member, the *D. melanogaster* gene *60A*, is also present in only one copy in each species. Comparison of homologous sequences from *D. melanogaster*, *S. americana*, and *H. sapiens*, representing roughly 900 million years of evolutionary distance, reveals significant constraint on sequence divergence for both *dpp* and *60A*. In the signaling portion of the *dpp* protein, the amino acid identity between these species exceeds 74%. Our results for the TGF-β superfamily are consistent with current hypotheses describing gene duplication and diversification as a frequent response to high levels of selective pressure on individual family members.

Key words: TGF-β superfamily — *decapentaplegic* gene — *Drosophila melanogaster* — *Schistocerca americana* — Gene comparison

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Introduction

During development, the process of pattern formation faithfully reproduces in every individual the predictable arrangement of organs and tissues which characterize its species. Over the last decade, numerous studies have implicated secreted signaling molecules of the transforming growth factor-β (TGF-β) superfamily in important patterning events. These include mesoderm induction in *Xenopus*, regression of the Mullerian ducts in early human male development, skeletal patterning in embryos and bone remodeling in adults by numerous mammalian TGFβs (bone morphogenesis proteins: BMPs) and dauer larvae formation in *Caenorhabditis elegans* (reviewed in Kingsley 1994).

Members of the TGFβ superfamily of signaling proteins share a C-terminal fragment of roughly 110 amino acids which, when cleaved from the propolypeptide, forms the active ligand (reviewed in Massagué 1990). The TGFβ superfamily has been divided into several subfamilies based upon sequence similarities in the ligand region, revealing extensive evolutionary conservation. The largest subgroup, referred to as the *dpp*/BMP subgroup, includes the *D. melanogaster* genes *dpp*, 60A, and *screw* and at present 12 vertebrate genes including the mammalian BMPs (Arora et al. 1994). Within this subgroup, the most similar molecules are *dpp* and *H. sapiens* BMP2 and BMP4. The ability of these BMPs to rescue *dpp* null mutations (Padgett et al. 1993) and the demonstration that *dpp* can induce bone formation (Sampath et al. 1993) led to the proposal that *dpp* is homologous to both of these *H. sapiens* genes. Genetically and developmentally distinct from *dpp*, the *D. melanogaster* 60A gene also appears to be highly conserved; it is more

similar to *H. sapiens* BMP5, BMP6, and BMP7 than to the other *D. melanogaster* genes (Wharton et al. 1991).

In *D. melanogaster, dpp* is a complex gene which is expressed in highly localized patterns in many tissues and phases of the life cycle. *dpp* is required for proper pattern formation during several developmental stages. These include the determination of the dorsal ectoderm along the dorsal-ventral axis during early development (Irish and Gelbart 1987), larval gut differentiation during later embryogenesis (Segal and Gelbart 1985), and proximal-distal development of adult appendages (Spencer et al. 1982).

The fruit fly *D. melanogaster* is a holometabolous insect which utilizes larval and pupal forms between the embryonic and adult stages of its life cycle. The grasshopper *S. americana* is a hemimetabolous insect which hatches from the egg in a form very similar to that of the adult without using larval or pupal stages (Bentley et al. 1979). In addition to these dramatic life-cycle differences, the process of segmentation during embryonic development is also quite distinct in these insects. *D. melanogaster* is a "long germ band" insect which simultaneously subdivides its embryo to generate segments. *S. americana* is a "short germ band" insect which generates segments sequentially from rostral to caudal (Patel et al. 1992). *D. melanogaster* and *S. americana* belong to superorders which last shared a common ancestor during the Devonian period 350–400 million years ago (Hennig 1981).

To improve our understanding of the evolutionary diversification of the TGF-β superfamily we identified *dpp* in *S. americana.* Genomic analyses indicate that both insects contains only one copy each of *dpp* and 60A, notwithstanding a 60-fold difference in genome size. The numerous counterparts of both genes found in *H. sapiens* therefore reflect multiple TGF-β duplication events in the chordate lineage. Comparison of partial sequences from *dpp* and 60A from *D. melanogaster* with their homologs in *S. americana* and *H. sapiens* reveals that TGF-β family members evolve in a clocklike manner with the ligand region of each gene undergoing relatively little change. Our results suggest that duplication and diversification of specific developmental functions such as intercellular signaling molecules are correlated with increasing pattern complexity and represent a frequent mechanism of phenotypic change in the evolution of multicellular organisms.

Materials and Methods

Identification of S. americana dpp. 540,000 phage from a λgt11 cDNA library constructed from *S. americana* staged at 40% of development (Snow et al. 1988) were screened at low stringency (hybridization conditions—30% formamide, 5 × SSC [SSC: 0.15 M NaCl, 0.15 M sodium citrate], 1% SDS, 1% BLOTTO [BLOTTO: 10% Carnation nonfat dry milk] at 42°C; wash conditions—2 × SSC, 0.1% SDS at 50°C). An 850-bp subclone (850PN) from *D. melanogaster dpp* containing the highly conserved TGF-β region was used as a probe. A single positive plaque was identified and purified. The phage insert was subcloned and restriction mapped, and the cross-hybridizing region was sequenced. *S. americana* genomic Southern blots using this clone (161.8R1) were completed using standard stringency (hybridization and washes as above except with 50% formamide and 0.1 × SSC, respectively; Sambrook et al. 1989).

Northern Analysis. Pods of eggs were collected from a colony of *S. americana* maintained in the Biological Laboratories at Harvard University. Single embryos from each pod were staged according to the criteria given by Bentley et al. (1979). The remaining eggs were dechorionated in 50% clorox and quick frozen in liquid nitrogen. Total RNA was prepared from each stage by the method of Chomczynski and Saachi (1987) using the Ultraspec RNA Isolation System (Biotecx Laboratories, Houston). Poly A^+ RNA was isolated by the batch method and 10 μg from each stage was analyzed with ^{32}P-labeled riboprobes transcribed from 161.8R1 and *S. americana* fasciclin I (as a control for RNA loading and developmental stage) according to Newfeld et al. (1991).

5′ RACE. Using the 5′ RACE System (Gibco BRL, Gaithersburg; developed by Loh et al. 1989), 1-μg aliquots of poly A^+ RNA from embryos staged at 30–35% of development were reverse transcribed using a *S. americana dpp*–specific antisense primer (Hop5′.1; 5′-GTGAGCTGCGTCGGTATACAG-3′). Upon completion of RNAse H treatment and terminal transferase addition of an oligoC tail to the 5′ end of the cDNA, a polymerase chain reaction (PCR) containing a nested *S. americana dpp*–specific antisense primer (Hop5′.2; 5′-TTCGGCACGAGGCCAGGGTTC-3′) and an anchor primer (provided by Gibco BRL) was completed under the following conditions: 2.5 mM $MgCl_2$, 200 μM dNTP mix, 0.4 μM of each primer, 1 × PCR buffer, and 5 units of Taq DNA polymerase (Promega, Madison). Cycling parameters included a 5-min "hot start" at 94°C prior to enzyme addition at 80°C followed by 35 cycles of 94°C for 1 min, 62°C for 1 min, and 72°C for 2 min, and concluding with an incubation at 72°C for 10 min. Gel purification of a roughly 550-bp product which hybridized to *S. americana dpp* subclone 161.8R1 on Southern blots was followed by secondary PCR with a third nested *S. americana dpp*–specific antisense primer (Hop5′.3; 5-CACCACTTTGGACTCTTCATG-3′) and a universal amplification primer (provided by Gibco BRL). The same reaction and cycling conditions were used except that the annealing temperature was increased to 66°C. The reamplified RACE product was cloned by cutting with *Spe*I (a site is contained in the universal amplification primer) and *Eco*RI (a site exists in *S. americana dpp*). This clone (560ks) was sequenced and nucleotide sequences from *S. americana dpp* (a composite of 161.8R1 and 560ks), *D. melanogaster dpp* (Padgett et al. 1987), and *H. sapiens* BMP4 (Wozney et al. 1988) were aligned and compared as described in Newfeld et al. (1993).

Identification of S. americana *60A.* PCR using the degenerate oligonucleotide primers V_1 and V_2 was performed as described in Wharton et al. (1991). *S. americana* PCR products were cloned, numerous independent clones were sequenced, and sequences were aligned with *D. melanogaster dpp* or 60A. Clones containing *S. americana* 60A were then aligned and compared with *D. melanogaster* 60A and *H. sapiens* BMP5 as described above.

Results and Discussion

Identification of S. americana dpp *and Its Transcripts*

A low-stringency screen of a cDNA library constructed from staged *S. americana* embryos, predicted to express *dpp* by analogy with *dpp* expression during *D. melano-*

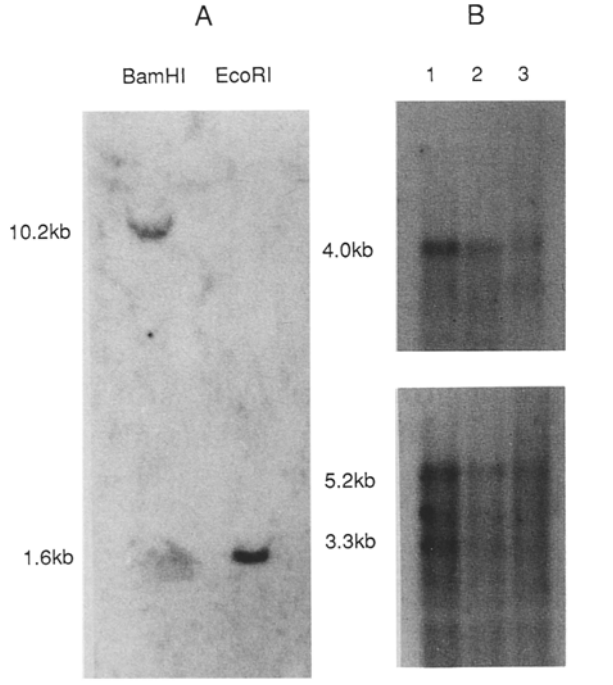

Fig. 1. Characterization of *S. americana dpp* genomic DNA and expression during limb development. **A** An autoradiogram of a filter containing 40 μg of *S. americana* genomic DNA digested with the indicated restriction enzyme and hybridized with *dpp* cDNA clone 161.8R1 at standard stringency. A single hybridizing restriction fragment is seen in each lane. The size of hybridizing fragments, as determined with reference to lambda DNA cut with *Hin*dIII (data not shown), is indicated. **B Upper panel.** An autoradiogram of a filter containing 10 μg of size-fractionated poly A^+ RNA from *S. americana* embryos staged at 30–35% of development (*lane 1*), 35–40% (*lane 2*), and 40–45% (*lane 3*), corresponding to the period of limb development in the embryo. The filter was hybridized with *S. americana dpp* clone 161.8R1. A single transcript is detected in each stage which appears to diminish in abundance with time. **Lower panel.** The same filter stripped and reprobed with *S. americana* fasciclin I as an approximate control for embryonic staging and RNA loading. The size of hybridizing transcripts, as determined with reference to a 0.24–9.5-kb RNA ladder (data not shown), is indicated.

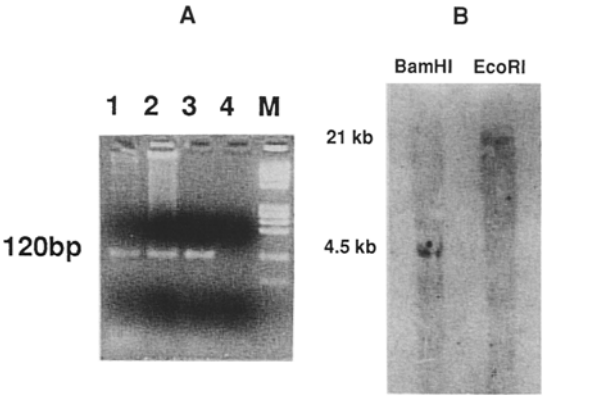

Fig. 2. PCR amplification and genomic analysis of an *S. americana* 60A homolog. **A** An agarose gel containing 10% of PCR reactions designed to amplify *S. americana dpp* and 60A. (See Materials and Methods.) The reaction in *lane 1* contained *D. melanogaster dpp* plasmid DNA as a control template (850PN; 20 ng). The *lane 2* reaction contained *S. americana* genomic DNA as template (1 μg). The *lane 3* reaction contained *D. melanogaster* 60A plasmid DNA as a control template (cDNA16; 1 ng). The *lane 4* reaction contained no template DNA and *lane 5* contains the molecular size marker ϕX174 cut with *Hae*III. The size of the amplified products is indicated and corresponds to the expected size based on *D. melanogaster dpp* and 60A sequences. **B** An autoradiogram of the filter from Fig. 1A, hybridized with a clone derived from the *S. americana* PCR product shown in *lane 2* of panel **A.** The cloned probe contained a portion of the *S. americana* 60A gene. A single hybridizing fragment is seen in each lane and the hybridizing fragments are clearly distinct from those identified by *S. americana dpp.* (See Fig. 1A.)

gaster development, identified a single clone. Subsequent subcloning and sequencing of the cross-hybridizing region revealed the C-terminal 150 bp from the ligand domain of *S. americana dpp.* Further sequencing demonstrated that the remainder of the clone comprised 1.6 kb of 3′-untranslated sequence without any poly A^+ tail. Rescreening the library identified numerous phage, all identical to the original clone. A genomic Southern to *S. americana* DNA cut with *Eco*RI (as shown in Fig. 1A) shows a single hybridizing fragment apparently identical in size to the cDNA. As a result of these observations, we hypothesized that the *S. americana dpp* cDNA was truncated at both ends by naturally occurring *Eco*RI sites during library construction. The existence of one of these *Eco*RI sites in *S. americana dpp* was positively confirmed by 5′ RACE. Northern analysis (Fig. 1B, upper panel) of *dpp* expression in staged *S. americana* embryos spanning the period of limb development (according to Bentley et al. 1979) revealed a single major transcript of 4.4 kb. In contrast, the expression pattern for *D. melanogaster dpp* during limb development (pupal stages) shows three major transcripts in the 3.5–4.5 kb range (St. Johnston et al. 1990).

The presence of two *dpp* homologs in *H. sapiens* (BMP2 and BMP4) suggested that the 60-fold-larger genome of *S. americana* (6×10^9 bp vs 1×10^8 bp in *D. melanogaster;* Wilmore and Brown 1975), which is twice as large as the *H. sapiens* genome, might contain multiple *dpp* homologs. However, the genomic Southern of *S. americana dpp* (Fig. 1A) shows only a single band with both *Eco*RI and *Bam*HI suggesting the existence of a single *dpp* locus in *S. americana.*

The evolutionary history of *dpp* is very similar to the history of another *D. melanogaster* TGF-β family member—60A (Wharton et al. 1991). 60A is developmentally distinct from *dpp;* its pattern of expression shows no overlap with *dpp* and its mutant phenotypes do not resemble *dpp* mutant phenotypes (K. Wharton, pers. comm.). 60A is more closely related to the *Homo sapiens* TGF-β proteins BMP5, BMP6, and BMP7 than to *D. melanogaster dpp,* suggesting that *dpp* and 60A are the result of an ancestral TGF-β duplication that preceded the divergence of arthropods and chordates (Wharton et al. 1991). 60A also appears to have a single homolog in *S. americana.*

The amplification of portions of *S. americana dpp* and 60A by degenerate oligonucleotide PCR is shown in Fig.

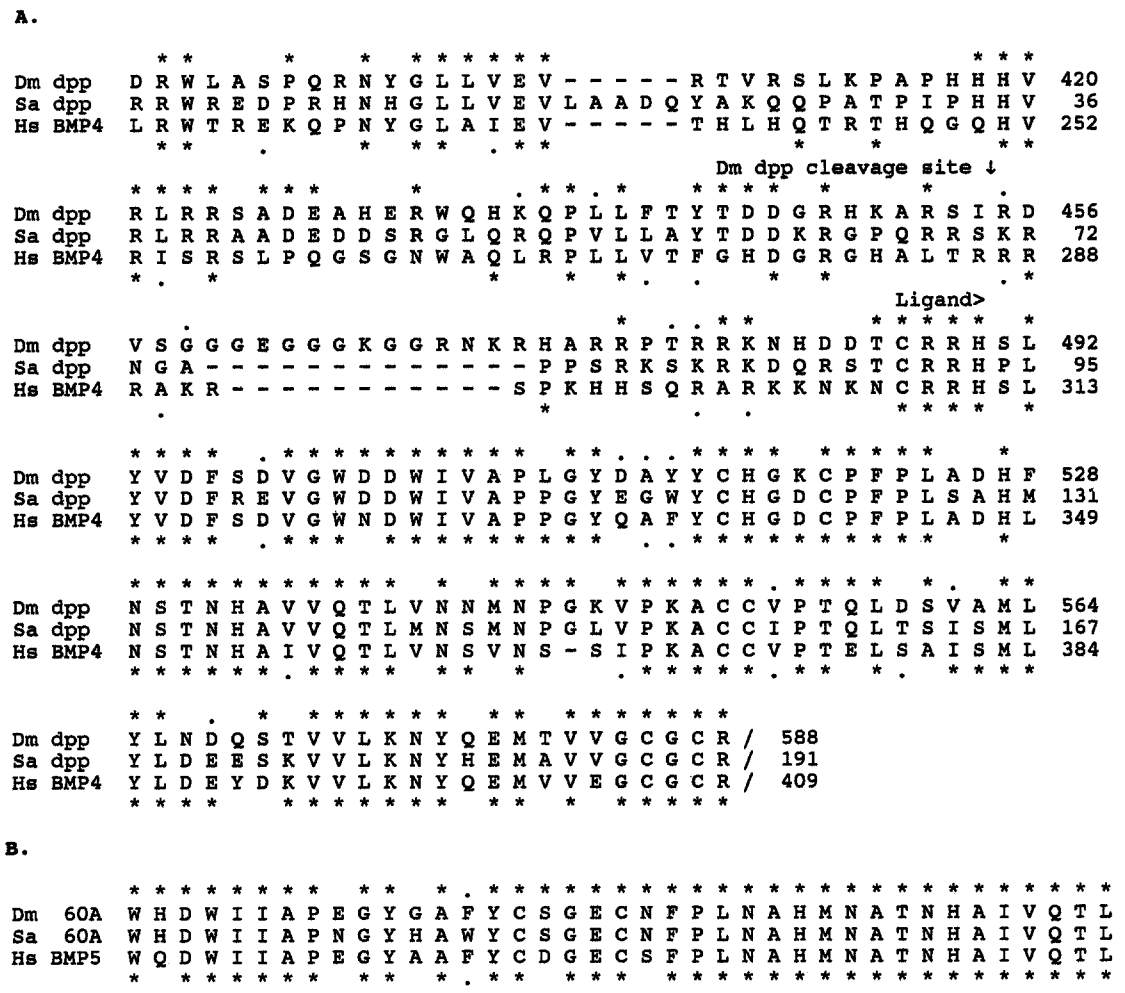

Fig. 3. **A** Alignment of the inferred partial amino acid sequences from *D. melanogaster dpp, S. americana dpp,* and *H. sapiens* BMP4. The amino acid sequences (in *one-letter code*) deduced from *D. melanogaster dpp* (Padgett et al. 1987, nucleotides 2358–2954; 597 total), *S. americana dpp* (nucleotides 1–573; 573 total), and *H. sapiens* BMP4 (Wozney et al. 1988, nucleotides 1058–1629; 576 total) cDNA sequences are aligned. For *S. americana dpp,* amino acid 1 corresponds to the first codon in the open reading frame of 5′ RACE clone 560ks. (See Materials and Methods.) For *D. melanogaster dpp* and *H. sapiens* BMP4 previously published amino acid numbers are utilized. The amino acids are *numbered* consecutively for each species and indicated at the *right margin. Dashed lines* indicate gaps required in the alignment to demonstrate maximum identity between the sequences. An *asterisk below* the alignment indicates an identical amino acid in *S. americana dpp* and *H. sapiens* BMP4. An *asterisk above* the alignment indicates an identical amino acid in *S. americana dpp* and *D. melanogaster dpp.* A *dot* above or below the alignment indicates a conservative substitution between the respective species, based on the biochemical similarity of the amino acids: D/E, K/R/H, N/Q, S/T, I/L/V, F/W/Y, and A/G (Smith and Smith 1990). The propolypeptide cleavage site for *D. melanogaster dpp* is noted (Panganiban et al. 1990). The first cysteine of the C-terminal region diagnostic for the TGF-β family is indicated by the designation *ligand.* Variability in the location of cleavage sites between different TGF-β family members results in significant divergence prior to the first cysteine. Only the TGF-β diagnostic region is considered as the ligand in the analysis. **B** Alignment of the inferred partial amino acid sequences from *D. melanogaster* 60A, *S. americana* 60A, and *H. sapiens* BMP5.

2A. Cloning the *S. americana* PCR products and sequencing numerous independent subclones identified only a single *S. americana* homolog of *dpp* and 60A. This strategy has also identified *dpp* and 60A homologs in the mosquito *Anopheles gambiae* (V. Kumar, pers. comm.) and the medfly *Ceratitis capitata* (L. Zwiebel, pers. comm.). Figure 2B shows the Southern blot from Fig. 1A hybridized with an *S. americana* 60A subclone. As with *S. americana dpp* a single band is seen in each lane, yet the 60A hybridizing fragments are clearly distinct from those hybridizing to *dpp.* The genomic data, both Southern blot and PCR, suggest that the BMP2/BMP4 duplication and the BMP5/BMP6/BMP7 duplications occurred after the split of arthropods and chordates. (The phylogenetic relationship of *D. melanogaster, S. americana,* and *H. sapiens* is diagrammed in Fig. 4.) Thus, it appears that the ancestral TGF-β duplication which created *dpp* and 60A was followed by additional duplications of each locus solely in the chordate lineage. These duplications are not simply the result of increasing genome size in this lineage as the *S. americana* genome is larger that the *H. sapiens* genome.

Table 1. Comparison of the deduced partial amino acid sequences of *D. melanogaster dpp, S. americana dpp,* and *H. sapiens* BMP4

	Percent identity			Percent similarity[a]		
	Overall	Proregion	Ligand	Overall	Proregion	Ligand
Dm dpp vs *Sa dpp*	58.6	37.5	76.7	66.0	45.5	83.5
Hs BMP4 vs *Sa dpp*	50.8	23.9	73.8	59.7	35.2	80.6
Hs BMP4 vs *Dm dpp*	51.8	27.1	74.8	58.3	39.6	76.7

[a] The percent similarity is the sum of the percent of identical amino acids and the percent of conservative amino acid substitutions between the listed species. Conservative amino acid substitutions are defined in the legend to Fig. 3

Comparison of S. americana dpp, D. melanogaster dpp, *and* H. sapiens *BMP4 Inferred Amino Acid Sequences*

The combined inferred open reading frame of *S. americana dpp* contains part of the propolypeptide region as well as the complete C-terminal ligand domain. In Fig. 3A, the open reading frame is aligned with the homologous regions of *D. melanogaster dpp* and *H. sapiens* BMP4 and the amino acid sequences are compared. As expected, the greatest amounts of amino acid identity and similarity are seen in the ligand region.

Table 1 summarizes all pairwise comparisons of the sequences from these species. Note that in all cases *D. melanogaster* and *S. americana dpp* are more closely related to each other than either is to BMP4 even though the genome size of *H. sapiens* and *S. americana* are very similar. Quantifying the results from Fig. 3A, it is clear that the ligand region is more than twice as conserved between the species as the proregion in all cases. As shown in Fig. 4, note the clocklike rate for nonconservative amino acid substitution between each insect and *H. sapiens* (41.7% vs 40.3%). However, the difference between the insects and *H. sapiens* (roughly 41%) is only 17% larger than the difference between insects (34%) even though more than twice as much time has elapsed. This suggests that the selective pressure against amino acid substitutions in individual TGF-β molecules is quite strong once a certain threshold is reached.

A similar mode of amino acid divergence is seen when comparing 60A in these species. In the section of the ligand depicted in Fig. 3B, all pairwise comparisons between species demonstrate greater than 84% amino acid identity and 87% amino acid similarity. The insect species show the highest amount of conservation and also appear equidistant from the *H. sapiens* sequence.

In theory, gene duplication may occur in two ways—by the duplication of individual genes or through genome duplication (Li 1983). Results from our analysis suggest that the degree of sequence similarity and copy number of homologous genes do not correlate with genome size but with organismal complexity. The strong selective pressure against substitutions seen in these molecules suggests that gene duplication and the diversification of the new genes is necessary to create new functions. Thus, duplication and diversification of TGF-β genes may represent an efficient mechanism for generating morphological complexity. The finding that several other important signaling molecules in *D. melanogaster* have multiple vertebrate homologs (e.g., *wingless* and *hedgehog;* Echelard et al. 1993) implies that this strategy is frequently employed in the generation of increasing pattern diversity in the evolution of multicellular organisms.

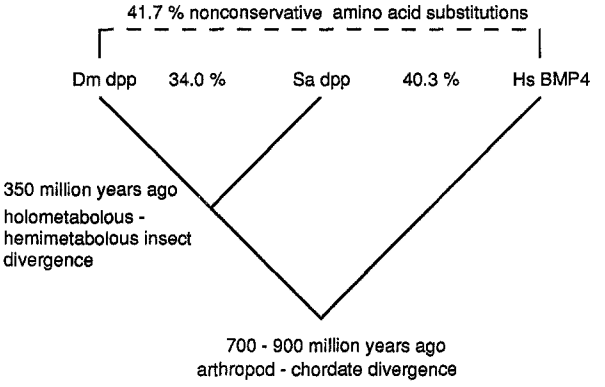

Fig. 4. Phylogenetic relationship between *D. melanogaster dpp, S. americana dpp,* and *H. sapiens* BMP4. The known ancestral relationship between *D. melanogaster, S. americana,* and *H. sapiens* (Field et al. 1988) is shown together with the amino acid differences of *dpp* and BMP4 from these species (calculated from Table 1). The clocklike nature of amino acid substitutions is clearly evident as each insect sequence is roughly equidistant from the *H. sapiens* sequence. However, the amino acid divergence between the insect sequences is nearly as great as the difference between each insect and *H. sapiens* sequence.

Note Added in Proof

The Gen Bank accession number for *S. americana dpp* is V23785.

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