Phosphoinositides could serve as specific membrane targets that bind proteins required for the formation of transport vesicles, such as the polypeptides of the adaptor complex that link clathrin to the cytoplasmic tails of certain transmembrane receptor proteins (for example, the mannose-6-phosphate receptor) (*28*). Vesicles from rat adipocytes that contain the glucose transporter also contain PI 4-kinase, which may regulate the transport (fusion) of these vesicles with the plasma membrane in response to insulin (*29*). In addition to its potential role in signaling cell proliferation, PI 3-kinase associated with receptor protein tyrosine kinases at the plasma membrane may also take part in the endocytosis and down-regulation (lysosomal degradation) of these receptors. In this way, the duration and the magnitude of the growth signal might be modulated. The association of Vps34p with the membrane appears to be mediated by the product of another *VPS* gene, *VPS15*. The *VPS15* gene encodes a membrane-associated protein kinase (Vps15) (*30, 31*) that can be chemically cross-linked to Vps34p (*21*). This raises the possibility that the Vps15 and Vps34 proteins may function together as components of a signal transduction complex that regulates intracellular protein sorting decisions.

REFERENCES AND NOTES

1. L. C. Cantley *et al.*, *Cell* **64**, 281 (1991).
2. J. M. Backer *et al.*, *EMBO J.* **11**, 3469 (1992).
3. S. Soltoff, S. Rabin, L. Cantley, D. Kaplan, *J. Biol. Chem.* **267**, 17472 (1992).
4. M. J. Berridge and R. F. Irvine, *Nature* **341**, 197 (1989).
5. M. Whitman *et al.*, *ibid.* **332**, 644 (1988).
6. D. L. Lips *et al.*, *J. Biol. Chem.* **264**, 8759 (1989).
7. L. A. Serunian *et al.*, *ibid.*, p. 17809.
8. C. Carpenter *et al.*, *ibid.* **265**, 19704 (1990).
9. F. Shibasaki, Y. Homma, T. Takenawa, *ibid.* **266**, 8108 (1991).
10. M. Otsu *et al.*, *Cell* **65**, 91 (1991).
11. I. D. Hiles *et al.*, *ibid.* **70**, 419 (1992).
12. C. A. Koch, D. Anderson, M. F. Moran, C. Ellis, T. Pawson, *Science* **252**, 668 (1991).
13. P. Hu *et al.*, *Mol. Cell. Biol.* **12**, 981 (1992).
14. C. J. McGlade *et al.*, *ibid.*, p. 991.
15. P. K. Herman and S. D. Emr, *ibid.* **10**, 6742 (1990).
16. S. Kornfeld and I. Mellman, *Annu. Rev. Cell Biol.* **5**, 483 (1989).
17. D. J. Klionsky, P. K. Herman, S. D. Emr, *Microbiol. Rev.* **54**, 266 (1990).
18. J. E. Rothman and L. Orci, *Nature* **355**, 409 (1992).
19. T. Stevens *et al.*, *Cell* **30**, 439 (1982).
20. T. R. Graham and S. D. Emr, *J. Cell Biol.* **114**, 207 (1991).
21. J. H. Stack, P. K. Herman, P. V. Schu, S. D. Emr, *EMBO J.*, in press.
22. K. R. Auger, C. L. Carpenter, L. C. Cantley, L. Varticovski, *J. Biol. Chem.* **264**, 20181 (1989).
23. G. Endemann, S. N. Dunn, L. C. Cantley, *Biochemistry* **26**, 6845 (1987).
24. Genetics Computer Group sequence analysis package; University of Wisconsin.
25. S. K. Hanks *et al.*, *Science* **241**, 42 (1988).
26. D. R. Knighton *et al.*, *ibid.* **253**, 407 (1991).
27. M. P. Sheetz and S. J. Singer, *Proc. Natl. Acad. Sci. U.S.A.* **71**, 4457 (1974).
28. B. M. Pearse and M. S. Robinson, *Annu. Rev. Cell Biol.* **6**, 151 (1990).
29. R. L. Del Vecchio and P. F. Pilch, *J. Biol. Chem.* **266**, 13278 (1991).
30. P. K. Herman *et al.*, *Cell* **64**, 425 (1991).
31. P. K. Herman, J. H. Stack, S. D. Emr, *EMBO J.* **10**, 4049 (1991).
32. T. Kunkel, *Proc. Natl. Acad. Sci. U.S.A.* **82**, 5463 (1985).
33. F. Sherman, G. R. Fink, L. W. Lawrence, *Methods in Yeast Genetics: A Laboratory Manual* (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, 1979).
34. M. Whitman *et al.*, *Nature* **315**, 239 (1985).
35. J. P. Walsh, K. K. Caldwell, P. W. Majerus, *Proc. Natl. Acad. Sci. U.S.A.* **88**, 9184 (1991).
36. L. J. Wickerham, *J. Bacteriol.* **52**, 293 (1946).
37. K. R. Auger *et al.*, *Cell* **57**, 167 (1989).

38. We thank members of the Emr lab for discussions and G. Huyer for his help in optimizing the PI 3-kinase assay. Supported by the Howard Hughes Medical Institute, a grant from the NSF (to S.D.E.), and by a fellowship from the Deutsche Forschungsgemeinschaft (to P.V.S.). S.D.E. is an investigator of the Howard Hughes Medical Institute.

8 October 1992; accepted 13 January 1993

Natural Selection and the Origin of *jingwei*, a Chimeric Processed Functional Gene in *Drosophila*

Manyuan Long* and Charles H. Langley

The origin of new genes includes both the initial molecular events and subsequent population dynamics. A processed *Drosophila* alcohol dehydrogenase (*Adh*) gene, previously thought to be a pseudogene, provided an opportunity to examine the two phases of the origin of a new gene. The sequence of the processed *Adh* messenger RNA became part of a new functional gene by capturing several upstream exons and introns of an unrelated gene. This novel chimeric gene, *jingwei*, differs from its parent *Adh* gene in both its pattern of expression and rate of molecular evolution. Natural selection participated in the origin and subsequent evolution of this gene.

How genes with novel functions evolve remains a fundamental and fascinating question. Gene duplications (*1*), exon shuffling, and processed genes (*2*) have been suggested as important sources of novel genes, but little is known about the evolutionary mechanisms or the participation of natural selection in their early history. Our analysis of the structure, expression, and evolution of a putative processed *Adh* pseudogene in *Drosophila* provided an opportunity to examine both the early molecular events and the evolutionary processes that created it. The *jingwei* (*jgw*) gene, as we named it (*3*), is a locus located on chromosome 3 in the *Drosophila* sibling species *D. teissieri* and *D. yakuba*. A part of *jgw* was initially observed to hybridize to the *Adh* probe (*4*). Further analysis suggested that in a single event, in the ancestor of the two species the *Adh* portion of this potential pseudogene was retrotransposed from an mRNA of the *Adh* locus on chromosome 2 (*5*). To understand the molecular population genetics of this potential pseudogene, we investigated its within-species DNA sequence variation. However, our results convince us that *jgw* is not a pseudogene and that the *Adh*-derived sequence is a part of a novel gene.

The *Adh*-derived portion was sequenced from ten *jgw* alleles of *D. teissieri* and of 20 *jgw* alleles of *D. yakuba* collected from natural populations (*6*). Figure 1 shows the distribution of nucleotide polymorphisms within species and the variation between species in a 765-bp segment that corresponds to the protein coding region of the *Adh* gene. Only one possible ancestral polymorphism was apparent (site 782). A summary of the DNA sequence variation in this region is presented (Table 1).

Unexpectedly, most polymorphisms were silent (eight out of ten in *D. teissieri* and 19 out of 21 in *D. yakuba*). If *jgw* were a pseudogene in which mutations had no phenotypic effect, most changes would be at replacement sites and there would be a reasonable frequency of stop codons (*7*). No new stop codons were present. Another prediction of the pseudogene hypothesis is that a pseudogene has a higher overall level of nucleotide variation. Estimates of DNA sequence polymorphism in *jgw* were similar to that found in the *Adh* gene (Table 1) and are typical of many functional genes in *Drosophila* (≈0.005) (*8*). A comparison of between-species divergence also revealed a significant bias toward silent versus replacement substitutions, and the degree of bias was smaller than for the within-species comparison. The bias was noted in the earlier study (*5*) but by itself was not large enough to convince the authors that this was not a pseudogene. In addition, insertions and deletions are abundant in the evolution of mammalian pseudogenes (*7*). In *jgw*, no length polymorphism or divergence was observed in the coding region (*9*). These molecular population genetic observations imply that the *Adh*-derived sequence is all or part of *jgw*, a new functional gene in *D. teissieri* and *D. yakuba*. This conclusion motivated our search

Section of Genetics, Section of Evolution and Ecology, and Center for Population Biology, University of California at Davis, Davis, CA 95616.

*To whom correspondence should be addressed.

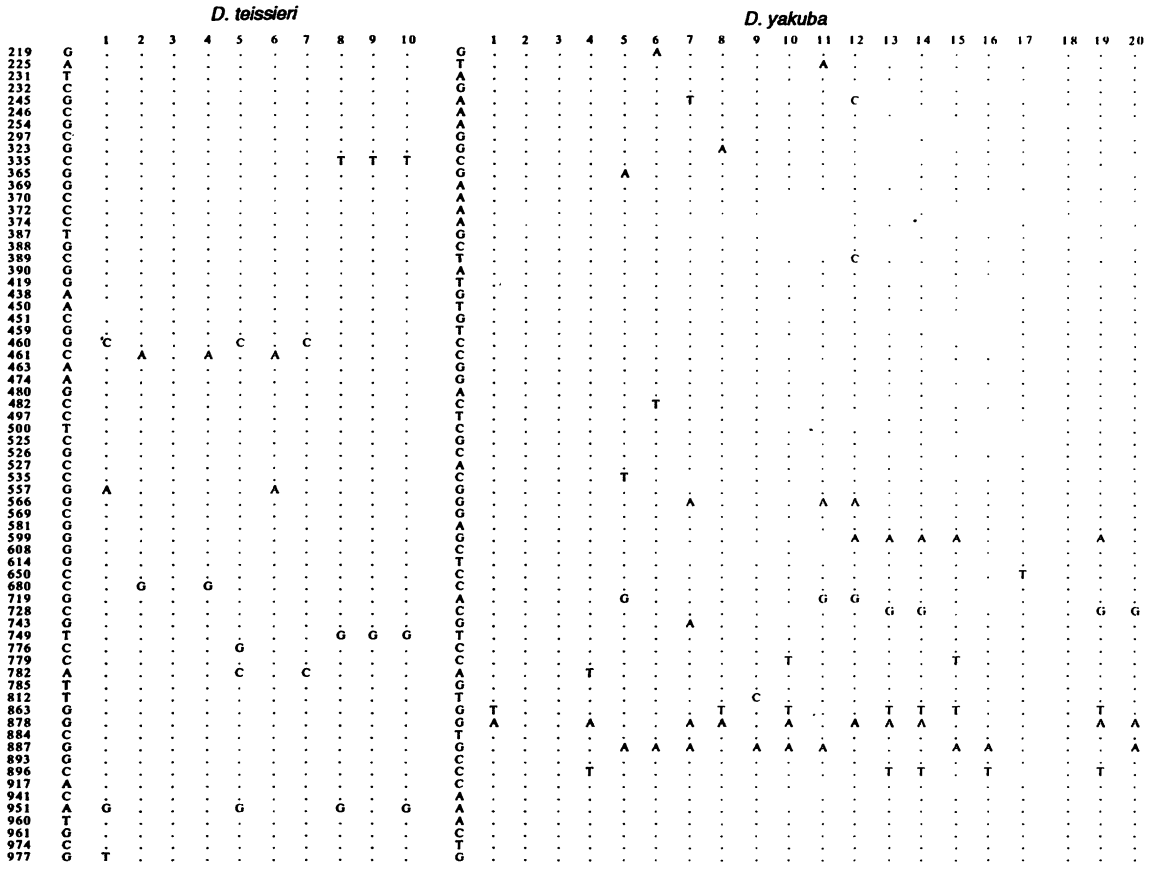

Fig. 1. Polymorphic and divergent differences in the *Adh*-derived portion of *jgw*. The nucleotide positions are indicated in the first column, numbered according to that reported from *D. yakuba* (*5*). The second and thirteenth columns show the consensus nucleotides. The dots indicate that the nucleotides are identical to those of the consensus. The first row contains the numbers of the sequenced alleles (10 from *D. teissieri* and 20 from *D. yakuba*).

for an RNA from *jgw* and a more detailed analysis of its structure.

Northern (RNA) analysis (*10*) of both total and polyadenylate [poly(A)]-selected RNAs from adults probed with *jgw* yielded only a single band that corresponded in size to that derived from the *Adh* gene (*11*). Because of the sequence similarity between the two genes and the abundance of the *Adh* mRNA, this result was not surprising. Two interpretations are possible. (i) The *jgw* mRNA may be detectably abundant but obscured by the *Adh* signal if its size is similar to that of *Adh*. (ii) The *jgw* mRNA may be so rare (or nonexistent) that it was undetectable by the Northern technique.

We used the polymerase chain reaction (PCR) in conjunction with reverse transcription (RT-PCR) (*12*) to identify RNAs with greater sensitivity and specificity than is possible with Northern analysis. Several regions can be found in which *Adh* and the *Adh*-derived portion of *jgw* differ by two or three contiguous substitutions. Under optimal PCR conditions, primers that contain these substitutions at their 3′ ends specifically amplified *jgw* RNA. A *jgw*-specific amplification product was obtained from total RNA extracted from both species. Sequencing these products confirmed the characteristic substitutions of the *jgw* gene in the 571-bp amplified fragment from *D. teissieri* and the 321-bp amplified fragment from *D. yakuba* (*13*). These results demonstrated the presence of *jgw*-derived RNA in both species.

The single band in the 5′ rapid amplification cDNA end (RACE) product and the identity of the two sequenced clones from this product indicated that *jgw* RNA has a distinct 5′ end in *D. teissieri* (*14*). In both species, *jgw* appears to have captured more than 180 bp of additional exon (or exons) in the 5′ direction (Fig. 2). The length of the 3′ end of *D. teissieri* *jgw* RNA is similar to that of *Adh* RNA. Figure 2 also shows the sequence of these products. By extending the *Adh* reading frame in the 5′ direction, the *D. teissieri* *jgw* appears to have acquired a new start codon and potentially encodes a hybrid protein with an additional 77 amino acids added to the *Adh*-derived protein. A preliminary analysis of *jgw* in *D. yakuba* indicated that it contains a similar structure.

To determine the structure of the genomic region (or regions) containing the exon (or

Table 1. Summary of the variation within and between species. The numbers of segregating (or polymorphic) sites are classified as replacement (R) and silent (S). Estimates of θ and π are measures of within-population variation in terms of the parameter $4N\mu$ per nucleotide, assuming the neutral theory of molecular evolution (*20*) where N is the effective population size and μ is the mutation rate for selectively equivalent nucleotides. The values of divergence between species (average divergence per nucleotide) are the results of averaging pairwise comparisons between the alleles from the two species minus average within-species differences, subject to the multiple substitution correction according to the Jukes and Cantor model (*28*). The standard deviations of θ and divergence estimates were calculated as described (*29*). *Adh* polymorphism data (19 of all the samples) of *D. yakuba* and *D. teissieri* *Adh* sequences are from (*5, 19*).

Gene	Segregating sites (n)		θ	π
	R	S		
	Polymorphism within species			
D. teissieri jgw	2	8	0.005 ± 0.003	0.005
D. yakuba jgw	2	19	0.008 ± 0.003	0.006
D. yakuba Adh	0	18	0.006 ± 0.003	0.006
	Divergence between species			
jgw	0.041 ± 0.009	0.127 ± 0.027		
Adh	0.007 ± 0.004	0.059 ± 0.018		

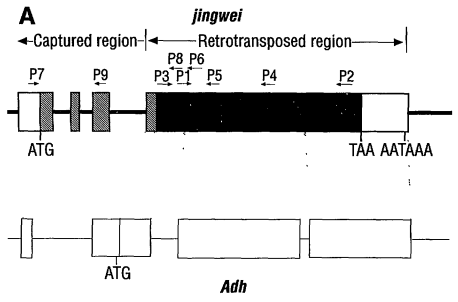

Fig. 2. Structure of the *jgw* transcription unit (*13*). (**A**) The structure of *jgw* deduced from the comparison of the sequenced *D. teissieri* RNA and genomic DNA. The four boxes show the four exons in *jgw*, and the solid and hatched portions of the boxes are the putative protein coding regions, *Adh*-derived and recruited, respectively. The open boxes are the putative untranslated portions. The lines among boxes are the three introns. The exon structure of the original *Adh* gene is also given, based on (*5*). The gray portions in four *Adh* exons are fused into one exon in the *jgw* gene. (**B**) Sequences of the 5′ regions of *jgw* from *D. teissieri* and *D. yakuba*. Introns are shown in lowercase letters. The codon ATG underlined in the *D. teissieri* sequence is the hypothetical start codon. The 3′ region of *D. teissieri jgw* is the same as that of the published sequence (*5*). An apparent polyadenylation signal, AATAAA, is at positions 1123 to 1128 of *D. teissieri*.

B ***D. teissieri* 5′ portion sequence of *jgw***

```
AGTATCAAAGTTTCATTGTATTAGAACAAACATTTCAATTTCGGCAAACATTACAAAATAAAAAAAAAAATCAAAAAATTTA
CTGTATTGTATTAACCAATTTGATTATAATGGCGCTTCGTCTTACAACCATTACTCTTCTAAAACGCACTCCTTTATTACTCGTGCCAAAGg
                                                                        Exon 1
tgagtctacaaaccctattagatcctgttcatatggtaattcattattaacaatgcgattcgcagTTAAAAGCAGCACCGACAACACGCACT
                                                  Intron 1                              Exon 2
CTACTACTAAGAAgtaagtaactcgtgggcaaaagtccaagtcagaattgcttatgtgcaaacccatGTCCTTTTCCAGGGAATCTGGTACC
                                                  Intron 2
GTTTTCAGATGATGATCCTTGTGTGTTCTCAAAGAAGGCCATCATGCACCATGGGGGGGtaagtactatatgatgagttgtctagaactcca
                                                  Exon 3
aaactcaagacttaccacctttgtcataaacctataaaaggattataaaagatatatatatatattattttcagTGCGGTTGTCAGTTG
                                                  Intron 3
CAGTTCAGCAGACCGGCTAGCAAGTGCGAGGCGTTTACCTTGTCCAACAAGAACGTG.....
                                                  Exon 4 (Adh-derived)
```

D. yakuba* 5′ portion sequence of *jgw

```
?.....?TCTTTCGACCATTGCTCTTCTGAAACGCACTCCTTTGTTACTCGTGCAAAAGgtgaggctaaaaatccctattagattctgttc
      Exon 1                                                                    Intron 1
atatggtaattcactattaacaatgccattcgcagTTAAAAGCAGCACAGCCAACACGCACTCTACTACTCTCTACTACAACAAgtaagtaa
                                                  Exon 2
ctcgtgggcaaaagtccaagtcaggattgctaagtgcaaacccatgtccttttacagGGAATCTGGTTCCGTTTTTAGGTGATGATTCTTGT
            Intron 2                                                        Exon 3
GTGTTCTCAAATAAGGCCATCATCCGCCATGGGGGGgtagtagtctagaactccaaaacttaccacatttgatataagcgattcaaatgaga
caaatacataactatccaggagatctgctccgggttctcttcttcatatcgatcatattaatattgtctcagtgcagttgtcagttgcag
                                                  Intron 3
ttcagcgagtaattgcatctctttaaatttacttatttgatcaaatcgacagAGCCAGTGCGACCAAAAAGAAGCGTTTTCC.....
                                                                        Exon 4 (Adh-derived)
```

exons) from which this new mRNA is derived, we carried out PCR-based genomic sequencing. Three introns (and three exons) were found 5′ to the *Adh*-derived exon in both species (Fig. 2). The size and positions of the three introns are similar in the two species, and the standard intron-splicing signal (GT-AG) was found in each case except for the *D. teissieri* second intron (GT-AT).

Because the structure of *jgw* is similar in both species, it seems likely that the inserted exons of *Adh* recruited the observed three 5′ exons (and introns) rather quickly. This interpretation is supported by the observation that no silent substitution accumulated in the *Adh*-derived portion of *jgw* before speciation, although several replacement substitutions were common to *jgw* in both species. This suggests that there was relatively little time between the insertion event and the subsequent molecular evolutionary events: the recruitment of the additional 5′ components into a functional transcription unit, the substitution of several amino acids, and the speciation.

In spite of similarities of structure and early common ancestry, *jgw* appears to have evolved distinct patterns of expression in the two species. Figure 3 shows the pattern of *jgw* expression at different development stages determined by quantitative PCR technique (*15*). Quantitative PCR provides at least a relative comparison of abundance and confirmed that the amount of *jgw* RNA was generally smaller than the amount of *Adh*. In *D. teissieri*, *jgw* showed sex-specific expression. The expected band was abundant in amplifications of RNA from adult males, whereas no band was present in reactions from RNA of other stages or adult females. However, in *D. yakuba* reactions with RNA from all stages showed less expression than was seen with *D. teissieri* RNA. Larval stages L1 and L2 and adult male RNA showed more expression than L3 and adult female RNA. Just as the protein sequences evolved rapidly, the sequences controlling the regulation of *jgw* have also evolved rapidly. Further analysis will determine if the species-specific patterns of expression are the result of the divergence of sequences in and around the *jgw* transcription unit or whether these expression patterns reflect divergence at more distant, trans-acting regulatory loci.

Whereas a distinct mRNA was expressed from *jgw* in each species, the conclusion that *jgw* is a functional gene also rests on the evolutionary interpretation of the polymorphism observed within each species. The extension of this evolutionary analysis in order to incorporate between-species divergence reveals that the early history of *jgw* was dominated by positive natural selection instead of the neutral mutation and genetic drift that are expected to characterize the dynamics of pseudogenes (*16*).

To explore the divergence of *jgw* in the period immediately after the *Adh* retrotransposition but before the split of *D. teissieri* and *D. yakuba*, we compared the *Adh*-derived portions of all *jgw* alleles with the available *Adh* alleles in *D. teissieri* and *D. yakuba* and with *Adh* alleles from various members of the *melanogaster* subgroup (Fig. 4). The fixed nucleotide substitutions that distinguished the *Adh*-derived portion of all *jgw* alleles from *Adh* in all alleles of *D. teissieri* and *D. yakuba* were assumed to have been fixed in the ancestral species after the insertion. Eight such sites exist at positions 339, 576, 600, 791, 831, 861, 918, and 954. Surprisingly, all eight substitutions were amino acid replacements; no silent substitutions were found. By incorporating the available *Adh* sequences of the other six members of the *melanogaster* subgroup, we found that all eight substitutions were unique mutations that were fixed in the *jgw* lineage. Assuming the replacement rate of substitution of 0.5×10^{-9} per nucleotide per year [calculated from *melanogaster* subgroup *Adh* data (*17*)], these eight substitutions (out of 572 possible replacement sites)

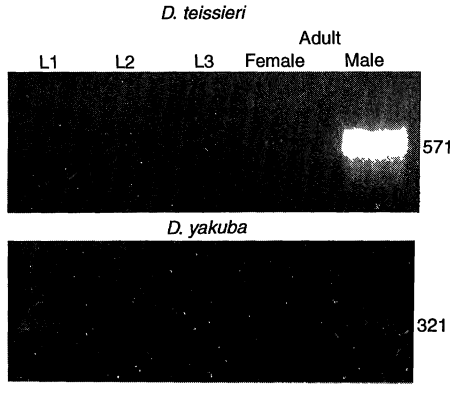

Fig. 3. Developmental pattern of *jgw* expression. The same method as described (*12*) was used to amplify *jgw* RNA from total RNA extracted from the different development stages. The cDNA product from 300 ng of RNA was added as a template for amplification because for the range from 100 ng to 1000 ng of total RNA, the yield of *jgw* RNA PCR products was observed to be linearly related to the template RNA concentration (*11*). PCR cycles (30) were conducted at 94°C (1 min) for denaturation, 60°C (2 min) for annealing, and 72°C (3 min) for polymerization. The products of the PCR reactions were electrophoresed on a 1% agarose gel and stained with ethidium bromide. More *Adh* RNA was amplified from the same cDNA preparations, which suggests that *Adh* is expressed in larger amounts than *jgw* (*11*). Molecular size markers are on the right in base pairs.

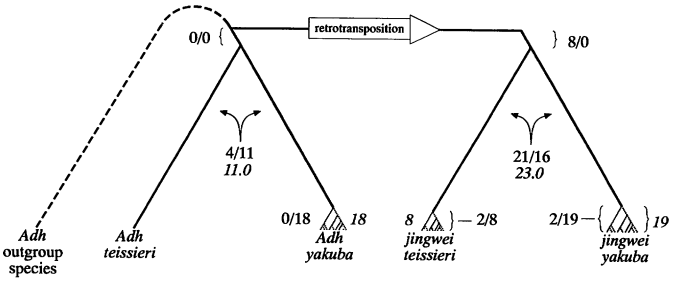

Fig. 4. Phylogenetic analysis of the evolution of the *Adh*-derived portion of *jingwei*. The topology reflects the assumed evolutionary relation between *jingwei* and *Adh* (*5*). The small (stippled) trees at the tips indicate that the detailed phylogenies of the alleles within species are unknown. Numbers presented as fractions are the numbers of (fixed or polymorphic) amino acid replacement differences over the numbers of (fixed or polymorphic) silent differences. The *Adh* outgroup includes the sequences of *D. melanogaster, D. simulans, D. sechellia, D. mauritiana, D. erecta*, and *D. orena*. These outgroup sequences were used to confirm that all functional *Adh* genes have the same DNA sequence for the eight codons that are distinct to *jingwei* (in both *D. teissieri* and *D. yakuba*) with one exception. At the eighth codon (nucleotide position 954) of *D. orena Adh*, there is a substitution that conferred an amino acid replacement. The italic numbers are the average numbers of silent substitutions and the numbers of silent polymorphisms under the HKA test, which also incorporates the standard assumptions of the neutral theory.

yielded an estimate of about 30 million years for the time of the last common ancestor of *jgw* and *Adh*. This conflicts with the age of the *melanogaster* subgroup, which is estimated to be 17 to 20 million years (*18*). The lack of silent substitutions implies that the insertion of *jgw* occurred close to the time of the speciation of *D. teissieri* and *D. yakuba*. The excess of replacement substitutions in the *jgw* line of descent between the time of its insertion and the speciation of *D. teissieri* and *D. yakuba* is consistent with the model that *jgw* responded to positive natural selection and evolved a new function.

This analysis of the early history of *jgw* leads us to examine its dynamics after the divergence of the two species. One method to explore the role of natural selection at a single locus is to compare the replacement and silent substitutions found between species with the replacement and silent polymorphism found within species (*19*). The variation in the *Adh*-derived portion of *jgw* within and between *D. teissieri* and *D. yakuba* is summarized (Fig. 4). A relative excess of (fixed) replacement substitutions over (fixed) silent substitutions between species (21:16) is apparent when compared to the proportion of replacement polymorphisms over silent polymorphisms within a species (4:27). These results are inconsistent with the null hypothesis in the test proposed (*19*), which incorporates the assumption of the selective neutrality (χ^2 = 13.6, $P < 0.001$). Therefore, adaptive protein evolution remained an important force in the evolutionary history of *jgw* after the separation of the two species. Further evidence supporting this view of the divergence of *jgw* in *D. teissieri* and *D. yakuba* comes from a comparison of the captured 5′ coding regions. Eleven out of the 15 differences in 153 alignable coding sites cause amino acid replacements.

Neutral allele theory considers polymorphism to be a transient phase of molecular evolution (*20*). Under the assumptions of this theory, the level of the between-species divergence at different loci is positively correlated with the level of within-species polymorphism. This idea is embodied in a simple, two-by-two statistical test (HKA test) (*21*) that calculates the expected amounts of polymorphism and divergence at two loci and can indicate whether the observed amounts are consistent with the neutral theory. Because we determined that there is an excess of replacement substitutions, we directed our attention to the silent variation in *jgw* and *Adh*. Figure 4 shows the observed numbers of silent substitutions and polymorphisms in the *Adh*-derived portion of *jgw*. Whereas their within-species polymorphism is comparable, the between-species divergence of *jgw* is twofold greater than that of *Adh* (χ^2 = 7.12, $P < 0.01$).

The *Adh* gene in these two species has a very biased codon usage with a 84 to 87% G+C content at the third codon positions. Furthermore, the codon preference as shown by the within-species polymorphism data in *D. yakuba* (*19*) also shows a very high G+C content (85.7%). Nevertheless, the codon usage in the *Adh*-derived portion of *jgw* seems to be evolving to a smaller amount of G+C. The G+C contents at the third codon position summed over all silent polymorphism sites in the two species are 70.0% for *D. teissieri* and 62.4% for *D. yakuba*, respectively. The G+C content at the third codon position in the fixed replacement sites between the two species is 60.0%. This evolution of a moderate G+C content of third sites is consistent with the higher rate of silent divergence for *jgw* and the general observation of a negative correlation between the codon bias (and G+C content) and the rate of silent divergence (*22*).

The results presented reveal the molecular, genetic, and evolutionary mechanisms that participated in the origin of the chimeric processed functional gene *jgw*. The chimeric nature of the expressed product of this novel gene is unique among processed genes, including those few that are functional, such as the second gene for rat insulin, the human PGK gene, and other examples that have been interpreted as outcomes of retropositions (*2*, *23*). It has been proposed that retrotransposition can be a major cause of intron loss during evolution (*24*), as it was with *jgw*. The evolution of *jgw* also demonstrates that retrotransposition can be a source of new, intron-containing genes in eukaryotic evolution. It is also unique among instances of exon-shuffling (*25*) in that it clearly involved the retrotransposition of an mRNA. Experiments of cross-hybridization with a genomic Southern (DNA) blot and Northern analysis with the use of a probe generated from the captured portion of *jgw* indicated that the source of the captured portion of *jgw* was a duplication of an unrelated gene (*26*). The analysis of the early molecular evolution of *jgw* indicated that *jgw* was functional from the beginning and experienced strong adaptive evolution for what must have been a novel function. Prevailing theories of the origin of new genes assume initial relaxation of selection (*1*). Our results provide a contrary interpretation in which natural selection is present throughout the origin of a new gene.

REFERENCES AND NOTES

1. S. Ohno, *Evolution by Gene Duplication* (Springer-Verlag, Berlin, 1970); T. Ohta, *Genome* **31**, 304 (1989); J. B. S. Haldane, *The Causes of Evolution* (Longmans, New York, 1932).
2. W. Gilbert, *Nature* **271**, 501 (1978); G. F. Hollis, P. A. Hieter, O. W. McBride, D. Swan, P. Leder, *ibid.* **296**, 321 (1982); J. R. McCarrey and K. Thomas, *ibid.* **326**, 501 (1987).
3. In an ancient legend from China (*San Hai Jing*), Jingwei, a daughter of the Emperor Yande (first Chinese emperor 3000 B.C.), tragically drowned while swimming in the East China Sea. Jingwei was then reincarnated as a beautiful bird that drops stones and wood into the sea in an attempt to fill it, thus preventing others from drowning. We used the name "jingwei" because this gene avoided the usual fate of processed gene (death) and was "reincarnated" into a new structure with novel function.
4. C. H. Langley *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **79**, 5631 (1982).
5. P. Jeffs and M. Ashburner, *Proc. R. Soc. London, Ser. B* **244**, 151 (1991).
6. The sequences were completely determined from both strands by the dideoxyribonucleotide chain-terminating method [F. Sanger *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **74**, 5463 (1977)] on single-stranded DNA templates prepared directly from the PCR products [R. G. Higuchi and H. Ochman, *Nucleic Acids Res.* **17**, 5865 (1989); K. R. Saiki *et al.*, *Science* **239**, 487 (1988)]. The primers used in PCR are 5′-GACAGTGATATGAGATTGCCG-3′ and 5′-GGAAGAATGTGAGTGTGCTTCG-3′ for *D. teissieri* and 5′-CTCTAGAACACCAAAACTTACC-3′ and 5′-GCATACATATTTGTAAAATGCAAG-3′ for *D. yakuba*. Each sequence was from a single fly of a separate isofemale line that had been mated to its siblings for three generations. The two alleles or haplotypes in the five heterozygous flies were deter-

mined by the cloning and subsequent sequencing of the PCR products.
7. W.-H. Li, in *Evolution of Duplicate Genes and Pseudogenes*, M. Nei and R. N. Koehn, Eds. (Sinauer, Sunderland, MA, 1983), pp. 14–37.
8. C. H. Langley, in *Population Biology of Genes and Molecules*, N. T. Takahata and J. F. Crow, Eds. (Baifukan, Tokyo, 1990), pp. 75–91; M. Kreitman, in *Evolution at the Molecular Level*, R. K. Selander, A. G. Clark, T. S. Whittam, Eds. (Sinauer, Sunderland, MA, 1991), pp. 204–221.
9. Only two length polymorphisms are observed in the 3′ flanking region of *D. yakuba*.
10. J. C. Alwine *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **74**, 5350 (1977).
11. M. Long and C. H. Langley, unpublished data.
12. E. S. Kawasaki, in *PCR Protocols*, M. A. Innis *et al.*, Eds. (Academic Press, New York, 1990), pp. 21–27.
13. Total RNA was extracted from male adult flies (*D. teissieri*) and L1 larvae (*D. yakuba*) as described [D. A. Goldberg *et al.*, *Cell* **34**, 59 (1982)]. We treated the RNA with ribonuclease-free deoxyribonuclease to avoid potential DNA contamination during the PCR experiment. For RT-PCR, the *jgw*-transcribed RNA sequences were amplified from cDNA as described by Kawasaki (*12*) with the *jgw*-specific primers. The primers included the pair 5′-GGCACTCAATCCAAAGGTGTG-3′ (P1) and 5′-GCCCAAGTCCAGTTTCCAGAGT-3′ (P2) for *D. teissieri* and the pair 5′-TCCTTGAGCAACAAGAACGTAA-3′ (P3) and 5′-GTAGTTGACGGCGATGGTTGC-3′ (P4) for *D. yakuba*. The PCR products were cloned into Bluescript SK+ and sequenced by the dideoxyribonucleotide chain-terminating method.
14. *Drosophila teissieri* 5′ and 3′ ends of RNA were amplified from cDNA with the RACE technique (*27*). For the 5′ end of *jgw* RNA from *D. teissieri*, the primer used for primer extension was primer 5 (P5, 5′-GGTGGTCTCGGCAATGG-3′), which anneals to the RNA from both *jgw* and *Adh*. The cDNA generated afterwards was tailed with deoxyadenosine triphosphate. Three primers were used for the subsequent PCR reactions. They included two artificial primers (*27*) [dT17-adapter and adapter primers that are able to anneal to the artificially added poly(A) tail of cDNA] and *jgw*-specific primer 6 (P6, 5′-TCACATCGTAGGGGTAGAAGGTGACGCA-3′). For the 3′ end of *jgw* RNA in *D. teissieri*, the *jgw*-specific primer 7 (P7, 5′-CCAATTTGATTATAATGGCGCTTCG-3′) or P1 in conjunction with the dT17-adapter primer was used to amplify the 3′ portion of *jgw* RNA of *D. teissieri*. The 5′ end of *D. yakuba jgw* RNA was amplified from cDNA with P7 and *D. yakuba jgw*-specific primer P4. Genomic DNA was amplified with the use of P7 and a downstream primer; P6 was used for *D. teissieri* and primer 8 (P8, 5′-TCCAGACCAATGCCTCCCAGACCGGCAACGAAAATT-3′) for *D. yakuba*. The positions of the introns are determined by alignment of genomic and RNA sequences. Sequencing methods of the PCR products included the direct sequencing and the cloning-sequencing approaches as described (*6*).
15. J. Chelly, J.-C. Kaplan, P. Maire, S. Gautron, A. Kahn, *Nature* **333**, 858 (1988); J. Singer-Sam *et al.*, *Nucleic Acids Res.* **18**, 1255 (1990).
16. W.-H. Li, T. Gojobori, M. Nei, *Nature* **292**, 237 (1981).
17. D. T. Sullivan *et al.*, *Evol. Biol.* **24**, 107 (1990).
18. D. Lachaise *et al.*, *ibid.* **22**, 159 (1988).
19. J. H. McDonald and M. Kreitman, *Nature* **351**, 652 (1991).
20. M. Kimura, *The Neutral Theory of Molecular Evolution* (Cambridge Univ. Press, Cambridge, 1983); G. A. Watterson, *Theor. Popul. Biol.* **7**, 256 (1975); M. Nei and F. Tajima, *Genetics* **97**, 145 (1981).
21. R. R. Hudson *et al.*, *Genetics* **116**, 153 (1987).
22. E. N. Moriyama and T. Gojibori, *ibid.* **130**, 855 (1992); P. Sharp and W.-H. Li, *J. Mol. Evol.* **28**, 398 (1989).
23. M. B. Soares *et al.*, *Mol. Cell. Biol.* **5**, 2090 (1985); A. M. Weiner *et al.*, *Annu. Rev. Biochem.* **55**, 631 (1986); H.-H. M. Dahl *et al.*, *Genomics* **8**, 225 (1990); A. Ashworth *et al.*, *EMBO J.* **9**, 1529 (1990); J. Brosius, *Science* **251**, 753 (1991).
24. G. R. Fink, *Cell* **49**, 5 (1987).
25. R. L. Dorit, L. Schoenbach, W. Gilbert, *Science* **250**, 1377 (1990); L. Banyai *et al.*, *FEBS Lett.* **163**, 37 (1983).
26. We named this non-*Adh* parent gene of *jgw* as *Yande* (*ynd*) in consistency with the Chinese legend about *jingwei* (*3, 11*).
27. M. A. Frohman *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **85**, 8998 (1988).
28. M. Nei, *Molecular Evolutionary Genetics* (Columbia Univ. Press, New York, 1987), pp. 276–279; T. H. Jukes and C. R. Cantor, in *Mammalian Protein Metabolism*, H. N. Munro, Ed. (Academic Press, New York, 1969), pp. 21–132.
29. W.-H. Li and D. Graur, *Fundamentals of Molecular Evolution* (Sinauer, Sunderland, MA, 1991); R. R. Hudson, in *Oxford Surveys in Evolutionary Biology*, D. Futuyma and J. Antonovics, Eds. (Oxford Univ. Press, New York, 1990), vol. 7, pp. 1–40.
30. We thank J. Gillespie, A. Clark, M. Turelli, K. Burtis, and all members of our laboratory for helpful discussions and critical reading of the manuscript. D. Lachaise kindly provided the *D. teissieri* isofemale lines. We also thank P. Jeffs and M. Ashburner for sharing DNA sequences of the *Adh*-derived portions of *jgw* in *D. yakuba* and *D. teissieri* before they published these sequences (*5*). Supported by NSF (C.H.L.) and the Center for Population Biology of the University of California at Davis, a University of California at Davis Graduate Research Award, and a Jastro-Shields Research Scholarship (M.L.).

26 October 1992; accepted 4 February 1993

Modulation of Neuronal Migration by NMDA Receptors

Hitoshi Komuro and Pasko Rakic

The *N*-methyl-D-aspartate (NMDA) subtype of the glutamate receptor is essential for neuronal differentiation and establishment or elimination of synapses in a developing brain. The activity of the NMDA receptor has now been shown to also regulate the migration of granule cells in slice preparations of the developing mouse cerebellum. First, blockade of NMDA receptors by specific antagonists resulted in the curtailment of cell migration. Second, enhancement of NMDA receptor activity by the removal of magnesium or by the application of glycine increased the rate of cell movement. Third, increase of endogenous extracellular glutamate by inhibition of its uptake accelerated the rate of cell migration. These results suggest that NMDA receptors may play an early role in the regulation of calcium-dependent cell migration before neurons reach their targets and form synaptic contacts.

In the developing brain, most immature neurons migrate to their distant final destinations by extending their leading processes and translocating their soma through a terrain that is densely packed with previously generated neurons and their processes (*1*). This movement of immature neurons is essential for the establishment of normal cytoarchitecture, synaptic connectivity, and function in the brain (*2*). In the cerebellum, granule cells migrate from the site of their origin in the germinal external granular layer toward the internal granular layer along the elongated processes of Bergmann glial cells (*3*). Recently Komuro and Rakic have demonstrated that the rate of granule cell movement across the molecular layer in the cerebellum depends both on extracellular Ca^{2+} concentrations and on Ca^{2+} influx through N-type Ca^{2+} channels (*4*). However, the regulatory mechanism underlying this Ca^{2+}-dependent process remains unknown. We have now examined the role of ionotropic receptors—NMDA, non-NMDA, $GABA_A$, and $GABA_B$ (GABA is γ-aminobutyric acid)—in granule cell migration; these receptors are expressed by immature granule cells (*5*) and can directly and indirectly affect Ca^{2+} influx and intracellular Ca^{2+} concentrations (*5, 6*).

To examine whether NMDA, non-NMDA, $GABA_A$, and $GABA_B$ receptors play a significant role in the migration of granule cells, we used slice preparations of the developing mouse cerebellum stained with a lipophilic carbocyanine dye [1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate (DiI)] and a laser scanning confocal microscope (*7*). Postmitotic granule cells in slice preparations migrate from the external granular layer toward the internal granular layer (Fig. 1) (*8*). Antagonists to these receptors were added to the culture medium in separate experiments. Blockade of the non-NMDA subtype of glutamate receptors [that is, kainate and AMPA (L-α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid) receptors] by 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX) (*9*), $GABA_A$ receptors by bicuculline (*6*), and $GABA_B$ receptors by phaclofen (*10*) failed to alter the rate of cell migration (Fig. 2, A and B). However, blockade of the NMDA subtype of glutamate receptor by D-2-amino-5-phosphonopentanoic acid (D-AP5) (*11*) or (+)-5-methyl-10,11-dihydro-5*H*-dibenzo[*a,d*]cyclohepten-5,10-imine hydrogen maleate (MK-801) (*12*) significantly decreased the

Section of Neurobiology, Yale University School of Medicine, New Haven, CT 06510.