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Identification of genes from pattern formation, tyrosine kinase, and potassium channel families by DNA amplification

(polymerase chain reaction/human/*Caenorhabditis elegans*/homeobox)

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ABSTRACT **The study of gene family members has been aided by the isolation of related genes on the basis of DNA homology. We have adapted the polymerase chain reaction to screen animal genomes very rapidly and reliably for likely gene family members. Using conserved amino acid sequences to design degenerate oligonucleotide primers, we have shown that the genome of the nematode *Caenorhabditis elegans* contains sequences homologous to many *Drosophila* genes involved in pattern formation, including the segment polarity gene wingless (vertebrate *int-1*), and homeobox sequences characteristic of the Antennapedia, engrailed, and paired families. In addition, we have used this method to show that *C. elegans* contains at least five different sequences homologous to genes in the tyrosine kinase family. Lastly, we have isolated six potassium channel sequences from humans, a result that validates the utility of the method with large genomes and suggests that human potassium channel gene diversity may be extensive.**

Recent findings have confirmed the long-standing belief that gene duplication and divergence play a primary role in evolution. Homologs of many mammalian proteins have been found in invertebrates and even in single-celled eukaryotes. In addition, within individual species, many proteins have been grouped into families based on conserved functional domains. The discovery of large gene families raises important questions: For example, to what degree are the biological roles of gene family members conserved during evolution? To what extent does functional diversity arise by modifications of the genes themselves or by modifications in their pattern of expression? To address these questions, it is necessary to study a large number of gene family members both within single species and across various phyla.

Genes that encode family members have been identified in a variety of ways. The most direct of these approaches is low-stringency hybridization, a method that involves screening through large numbers of DNA clones for those that base pair with probe sequences (e.g., see refs. 1–4). Despite the success of conventional low-stringency screens, this method suffers from certain disadvantages: Spurious hybridization often results from G+C-rich sequences and random homologies, and clone verification normally involves laborious steps of subcloning and DNA sequencing. These drawbacks slow the progress of identifying gene family members and make it difficult to isolate gene homologs rapidly from other species where their study might prove fruitful.

We have adapted the polymerase chain reaction (PCR) to identify likely gene family members in a way that overcomes many weaknesses of conventional low-stringency screens. Degenerate oligonucleotide primers are designed based on conserved protein sequence motifs and then tested by PCR for their ability to use a given species of genomic DNA as a template for amplification. To choose the primers, two small segments of protein sequence conservation are identified within each protein family. All possible codons that potentially encode these short amino acid sequences are included in the oligonucleotide pool with few guesses about codon usage. As in other PCR experiments, the oligonucleotides hybridize with opposite strands of a genomic DNA template to prime the synthesis of nascent DNA, and the primers are positioned so that the newly synthesized DNA strands overlap (5, 6). Repeated cycles of synthesis, denaturation, and primer annealing result in an exponential amplification of DNA between the primer ends. The amplified fragments are cloned and sequenced to confirm their identities.

This strategy provides hybridization probes to isolate genomic or cDNA clones in high-stringency screens. In addition, it can provide valuable information about putative gene family members in a very short time. In contrast to conventional low-stringency screens, PCR screens avoid a great deal of rescreening, restriction mapping, and DNA sequencing at the initial stages of analysis. Although cDNA could also be used for PCR, the use of genomic DNA allows sequences in the genome to be sampled without any bias imposed by differences in the level or site of RNA expression.

Here we describe the identification of a wide range of conserved DNA sequences in the nematode *C. elegans* and in humans using this method.

MATERIALS AND METHODS

Oligonucleotide Primers. Oligonucleotides were from the University of California (San Francisco) facility and were used unpurified after a single precipitation with ethanol. Degeneracy of the primers varied from 64-fold to 1056-fold. *tyr-kin-2, hom-2, prd-2,* and *wg-2* had the 6-base-pair (bp) recognition sequence for *Hin*dIII appended at their 5′ ends, while *tyr-kin-1, hom-3, prd-1,* and *wg-1* included the 6-bp *Eco*RI recognition sequence. This strategy provided convenient sites for directional cloning. The other primers contained no extra sequence at their 5′ ends. In some cases (e.g., *tyr-kin-1* and *tyr-kin-2*), primers were designed to include more than one amino acid at specific positions. All primers included between 20 and 23 bases of coding sequence.

PCR Reactions. Primers were phosphorylated with T4 polynucleotide kinase (New England Biolabs) prior to PCR and were added at a final concentration of 10 μM to a mixture containing 10 mM Tris·HCl (pH 8.3), 50 mM KCl, 2 mM $MgCl_2$, 0.01% gelatin, 0.1 mM dNTPs (Pharmacia), 1 unit of *Thermus aquaticus* DNA polymerase (*Taq* polymerase) (Perkin–Elmer/Cetus), and 1 μg of genomic DNA. This mixture was incubated in a thermocycler for 40 cycles: 1 min at 94°C, 1 min at 45°C, and 2 min at 55°C.

Cloning PCR Fragments. After amplification by PCR, fresh dNTPs at 0.1 mM and 5 units of Klenow polymerase (BRL)

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Abbreviation: PCR, polymerase chain reaction.

were added to the mixture, which was incubated at room temperature for 1 hr. The mixture was phenol extracted, precipitated with ethanol, and resuspended in ligase buffer, 0.5 μg of phosphorylated *Eco*RI linkers (New England Biolabs), and 5 units of ligase (New England Biolabs). After overnight incubation at 14°C, the ligase was destroyed by heating at 70°C for 5 min. The mixture was made 1× in the appropriate restriction enzyme buffer and digested with restriction enzyme (*Eco*RI for *Huk* and *en-3/5* clones, *Eco*RI/*Hin*dIII for *tyr-kin, hom-2/3, wg-1/2,* and *prd-1/2* clones). The material was subjected to electrophoresis through 2% low-melting-temperature agarose (SeaKem, FMC) and the correct bands were excised and ligated into pIBI30 plasmid (IBI).

DNA Sequence Analysis. DNA sequences were determined by the dideoxynucleotide method using Sequenase (United States Biochemical) on double-stranded plasmid isolated from miniprep cultures. Because of the possibility of base substitutions introduced by PCR, the sequences should not be taken as perfectly faithful representatives of genomic DNA. However, in no case did we find a single nucleotide difference in the spacer regions among clones of a certain class.

RESULTS AND DISCUSSION

***C. elegans* Homologs of Genes Involved in Pattern Formation.** Many genes involved in anteroposterior pattern formation in *Drosophila* have highly conserved DNA sequences (7–9). For example, several classes of homeobox sequences have been identified within genes that subdivide and diversify the anteroposterior axis of *Drosophila*. Genes of the paired (*prd*) class, including the segmentation genes *prd,* gooseberry (*gsb*)-*1* and *gsb2,* possess homeoboxes with certain conserved features, whereas engrailed (*en*) contains a homeobox with different distinctive features. Homeotic genes, such as Antennapedia (*Antp*) and Ultrabithorax (*Ubx*), contain yet another class of homeobox. The *Drosophila* gene wingless (*wg*), which appears to function in cell–cell signaling as the pattern of individual segments develops does not contain a homeobox, but it encodes a protein that closely resembles the product of the vertebrate protooncogene *int-1* in several regions (10).

Relatives of many of these gene families have been found in other organisms. This raises the question of whether the functions of the genes—for example, their interactions in regulating pattern formation—are also conserved. In *C. elegans,* genes that control cell growth and differentiation can be analyzed genetically at the level of individual cells, providing a very detailed view of gene function. Previously, we showed that the *mab-5* gene of *C. elegans,* a homeotic gene required for pattern formation in the posterior body region of *C. elegans,* is structurally similar to *Drosophila* homeotic genes such as *Ant* and *Ubx* (11). This finding suggests the possibility that *Drosophila* and *C. elegans* anteroposterior patterning systems may be evolutionarily related and that other genes involved in patterning along the anteroposterior axis of *C. elegans* may have close relatives in *Drosophila.*

To look for *C. elegans* homologs of genes that participate in *Drosophila* anteroposterior pattern formation, degenerate primers were constructed based on protein sequences conserved among all or most members of a family. For *en, prd,* and *Antp* homeoboxes, we chose pairs of primers that could discriminate between different classes of genes (Fig. 1*A*). In the cases of *en* and *Antp,* single bands, slightly larger than expected, were generated by PCR on *C. elegans* genomic DNA. Sequence analysis of clones obtained from these bands suggested the presence of short introns in each segment. These intron sequences were A+T-rich, a characteristic of other *C. elegans* introns, and flanked by consensus splice sites. The coding regions exhibited strong amino acid conservation with the corresponding *Drosophila* sequences. For *prd,* a single band of the predicted size was observed following PCR. The sequence of a clone derived from this band displayed marked similarity to the corresponding *Drosophila prd* protein sequence.

To clone the *C. elegans* homolog of *wg,* degenerate primers were chosen based on two short segments of identity shared by all known members of the family (*int-1,* int-related protein, and *wg*) (Fig. 1*B*). The functional importance of these regions is unknown. PCR using *C. elegans* genomic DNA generated a single fragment slightly larger than expected. Again, the DNA sequence of the clone revealed the presence of a 53-bp sequence likely to be an intron. This predicted intron fell at exactly the same position relative to the coding sequence as introns in *int-1* and *wg*. Three amino acid residues within the spacer segment were conserved, but the sequence was otherwise quite divergent (however, see below).

Identification of *C. elegans* Tyrosine Kinase Sequences. The tyrosine kinase family is very large, with the sequences of nearly 40 members reported so far (16). The most intensively studied members are encoded by protooncogenes such as *src* and *abl*. These proteins are believed to participate in cell growth and differentiation, although their precise roles are unknown. Insight into the regulatory pathways within which these proteins function has been limited by the lack of rigorous genetic analysis. The identification of *src*-like and *abl*-like genes in *Drosophila* has opened one avenue for investigation (17–19). Another *Drosophila* gene, sevenless, has been shown to encode a tyrosine kinase that controls a specific choice of cell fate in the eye (20, 21).

Because of the potential value of studying the developmental functions of tyrosine kinases in individual cells, we have looked for *C. elegans* homologs of these genes. We constructed oligonucleotide primers based on two sequences, each seven amino acids long, conserved among several vertebrate and insect tyrosine kinase family members, particularly the *src* and *abl* relatives (Fig. 2*A*). One of these sequences is believed to participate in the recognition of tyrosine residues (16). The spacing between the conserved protein sequences is 11 amino acids and is constant among all known members of the tyrosine kinase family. A single band of approximately the correct size (86 bp) was obtained after amplification of *C. elegans* DNA. Twenty individual clones obtained from the 86-bp fragments were sequenced. The clones fell into six different categories: classes I–VI (Table 1). Primer sequences were not included in this classification since they varied from clone to clone even within a given category, presumably a result of mismatch priming during PCR. Class I clones were identical in DNA sequence to a region within a previously identified nematode gene that is similar to the vertebrate *abl* subfamily of tyrosine kinase genes (25). Class II–V clones all possessed residues that are particularly highly conserved among different tyrosine kinases; for example, all sequences included a phenylalanine at spacer position 7 and a serine at position 11 (Fig. 2*A*). A second conserved feature, the distance between the oligonucleotide primers (34 bp), was constant among all these classes. Thus, the class I–V clones likely represented bona fide tyrosine kinase genes in *C. elegans*. The single class VI clone was slightly larger than expected (96 bp instead of 86 bp), the reading frame between the two primers was shifted, and there was no sequence similarity to tyrosine kinases in the spacer region (data not shown). Thus, it was probably an artifact generated by the procedure.

Identification of Human Potassium Channel Sequences. The potassium (K^+) channel family, in contrast to tyrosine kinases and homeodomains, is not well characterized. However, extensive diversity of these channels has been inferred from electrophysiological experiments (26). K^+ channels play important roles in the nervous system. They are prin-

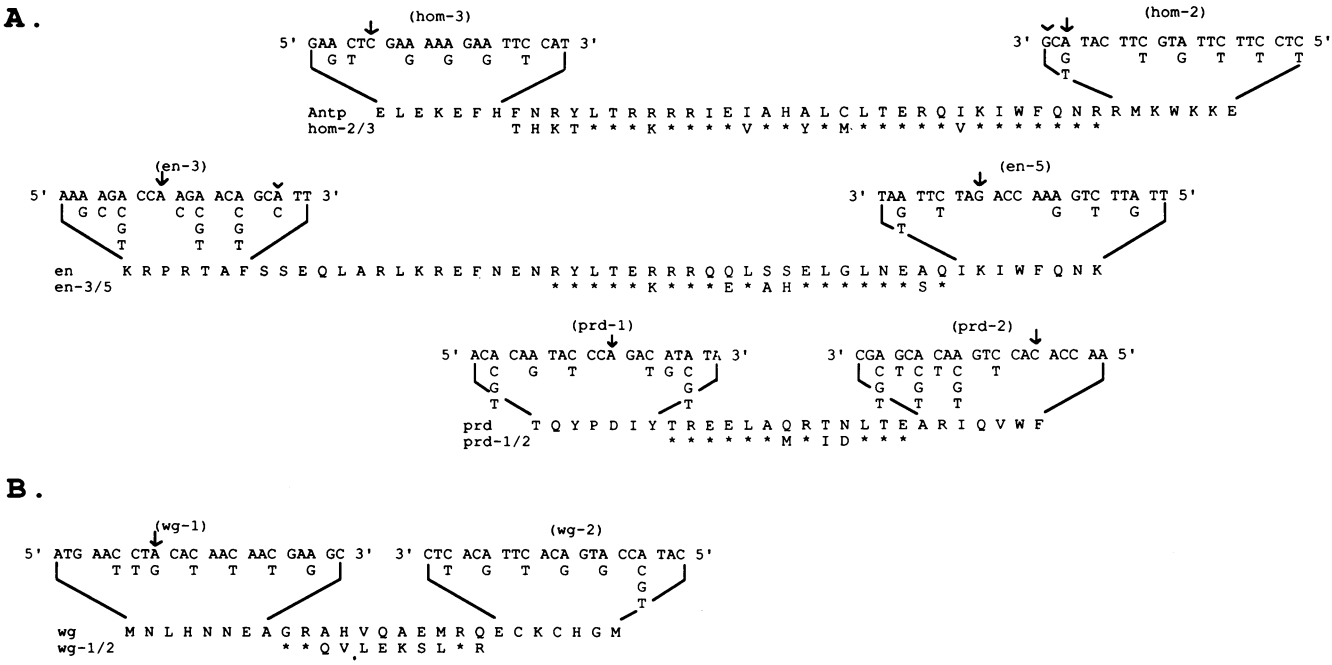

FIG. 1. Alignments of pattern-formation sequences. Spacer segments derived from PCR using *C. elegans* DNA are shown below the corresponding region of their *Drosophila* counterparts. Asterisks denote amino acids identical to those in the top sequence. The single-letter code is used. Conserved protein islands used for primer design are shown below the primer sequences. Arrows identify positions in the primer where up to 3 bases were omitted to reduce primer degeneracy. Thus, these positions are potential sites of mismatch between the primer and genomic template. (*A*) *Antp, en,* and *prd* homeodomain sequences are aligned with respect to each other to facilitate comparison of the residues conserved among all three classes and those that differ (3, 12–14). In two cases (*hom-2* and *en-3*), a related set of primers, differing only at or near the 3′ end, was also synthesized and tested. These positions are marked with carats. The en-3/5 sequence is abbreviated because the amino portion of the segment was lost during cloning due to the presence of an *Eco*RI site within the intron. (*B*) The *wg* sequence depicted above begins at position 214 in the protein (10). Spacer positions 1 and 2 are also conserved in all members of the family. Sequence analysis of clones obtained using the PCR fragment as probe confirm that this segment is part of a *C. elegans* protein that bears homology to *wg*, *int*-1, and *int*-related protein in several regions (10, 15).

ciple regulators of neuronal firing patterns, and they may underlie some forms of learning (27, 28). At least three classes are recognized: voltage dependent, inward rectifying, and calcium activated. Within each class, there is also much diversity; in all, >50 distinct types of K^+ channel have been detected. The molecular basis for this diversity is not yet clear. So far, only a single K^+ channel gene has been identified in *Drosophila,* mouse, and rat, although there is evidence that differential RNA processing may generate some channel diversity (22–24, 29–33).

We tested whether evidence for multiple K^+ channel homologs could be obtained by PCR, providing another possible explanation for channel diversity. Primers were chosen based on amino acid sequences conserved between a *Drosophila* K^+ channel and a putative K^+ channel from mouse (Fig. 2*B*). These sequences include portions of two hydrophobic presumptive membrane-spanning domains. A single band of approximately the predicted size (72 bp) was obtained after PCR by using human genomic DNA. Twenty individual clones obtained from the amplified human DNA were sequenced and compared with each other and with previously deduced channel sequences. The amplified spacer sequences of the clones fell into six classes (Table 1). However, all six types of sequence possessed several common features. All classes encoded a spacer segment of 10 amino acids, a length that is conserved among all known K^+ channels. In addition, each class contained residues conserved among all known K^+ channels: phenylalanine at position 7 in the spacer region, serine at position 9, and isoleucine at position 10 (Fig. 2*B*). Interestingly, class I spacer sequences were identical to the mouse sequence at the DNA level. Class II–VI nucleotide sequences were between 50% and 65% identical to the mouse DNA sequence within the spacer region, whereas amino acid identity varied from 40% to 50%. The amino acid differences displayed by the various sequences were in several cases either conservative replacements or changes that alter the net charge of the segment. Since voltage-sensitive ion channels are thought to use charged amino acid residues to detect changes in membrane voltage, the differences in net charge between different sequences could have functional consequences. None of the six shared sequences with any proteins other than K^+ channels, including other known ion channel proteins.

Evaluation of PCR Screening. The finding that PCR screening yielded amplified DNA fragments whose lengths and sequences were homologous to known members of a wide variety of gene families suggests that this method for cloning related genes is highly effective and specific. We did observe background bands of different sizes in some cases, particularly at lower temperatures and higher primer concentrations. Variation in G+C content of different primers and the possibility of mismatch priming probably accounted for the background. One tyrosine kinase clone (class VI) may have derived from such a background fragment. However, it was the only clone that we suspected to be artifactual. The chief advantages of this approach over conventional oligonucleotide screens are the stringency imposed by the requirement that two closely spaced oligonucleotides hybridize effectively and the relative ease of sorting through the cloned amplified material.

Given the number of different clones found to encode K^+ channel and tyrosine kinase sequences, it is worthwhile to consider some potentially misleading sources of diversity. First, PCR itself is known to introduce sequence changes at a detectable frequency; however, these nucleotide sequences were so divergent that it would be necessary to invoke an

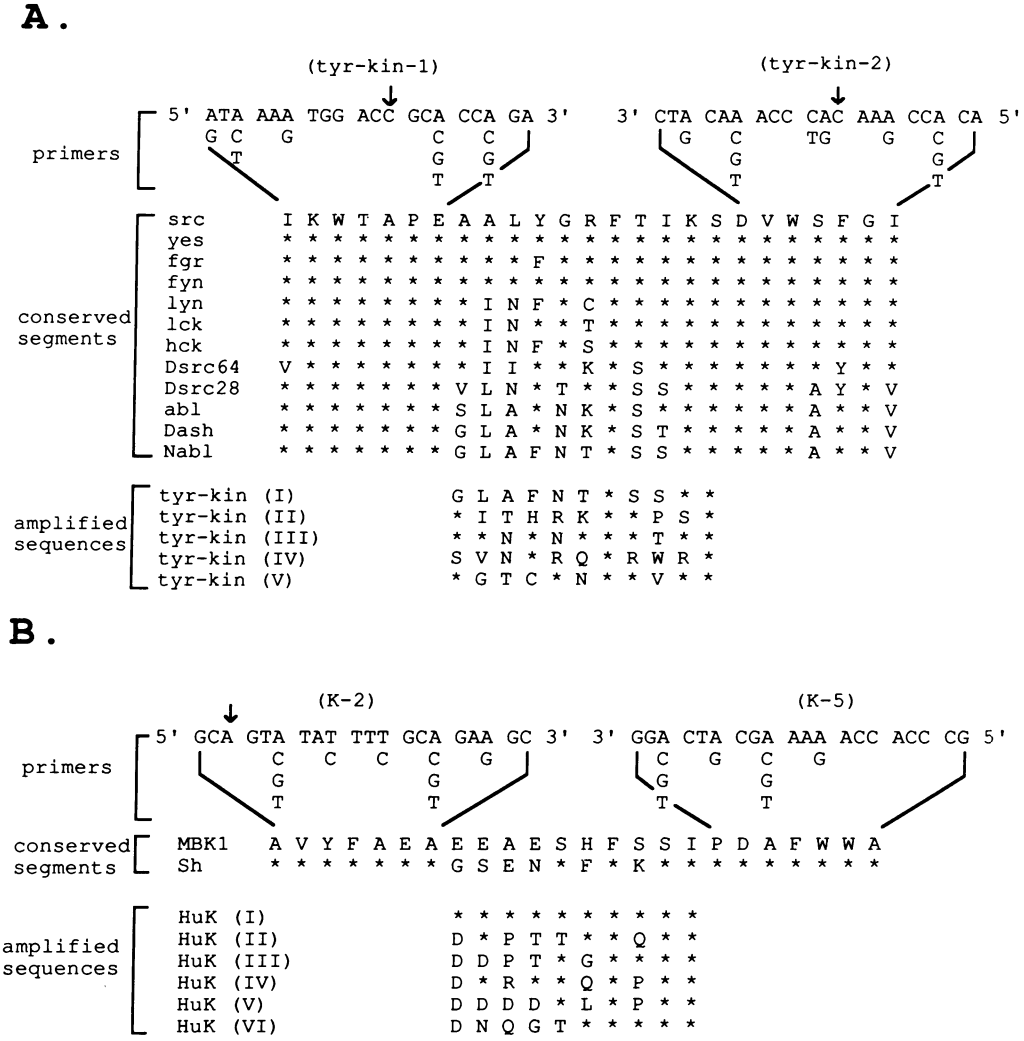

FIG. 2. Protein sequence alignments used in design of PCR primers for tyrosine kinase and K^+ channel primers. (*A*) Tyrosine kinase sequences are taken from Hanks *et al.* (16). Regions below the primer sequences correspond to their box VIII and box IX. All sequences are human except Dsrc64, Dsrc28, and Dash, which are *Drosophila* in origin, and Nabl, which is *C. elegans* in origin. Amino acid sequences of the spacer regions from the tyrosine kinase clones (inferred from DNA sequence) are shown below for comparison. The single-letter code is used. (*B*) K^+ channel sequences include a portion of the fourth hydrophobic domain and extend through part of the fifth hydrophobic domain. MBK1 is from mouse; Sh is from *Drosophila*. In *Drosophila*, mouse, and rat, K^+ channel proteins inferred from nucleotide sequences possess six hydrophobic domains and one sequence 21 residues long (S4) that is similar to a sequence in the sodium and calcium channel proteins (22–24). The spacer region between the primer sites encodes a segment that links the two hydrophobic domains.

error rate for PCR that is 70 to 500 times higher than previous estimates (5, 34). In addition, the nucleotide sequences of the spacer regions did not vary within a class, and the tyrosine kinase class I sequence was identical to the published *abl*-like sequence from *C. elegans*. Thus, it would be necessary to invoke a mechanism for generating errors that is sequence selective. Second, some of these sequences may have originated from genes that are only distantly related to tyrosine kinases or K^+ channels. However, given the primer hybridization, the primer spacing, and the presence of certain diagnostic conserved residues in the spacer region, these sequences possessed homology that extended over at least 24 amino acids. For the tyrosine kinases, at least part of this sequence appears to be associated with recognition of tyrosine substrates. Thus, strictly on the basis of homology, it seems most likely that these sequences encode tyrosine-specific kinases. For the K^+ channels, because this region is conserved only among known K^+ channels and is not present in other known channels, such as sodium and calcium channels, it would be surprising if these sequences were not derived from human K^+ channel genes. For the homeobox sequences, the presence of several conserved amino acid residues in the amino acid sequences reflects a high degree of similarity to other known homeodomains. Whether or not these sequences possess homology to known homeobox family members outside this region awaits more extensive characterization of the genes.

Table 1. Number of individual clone isolates per class of tyrosine kinase sequence or human K^+ channel sequence

	Class					
	I	II	III	IV	V	VI
Tyrosine kinase clones	7	7	3	1	1	1
Human K^+ channel clones	8	4	3	3	1	1

Twenty clones of each type (tyrosine kinase or K^+ channel) were sequenced. Note that genes with identical nucleotide sequences within the region that was analyzed would not be distinguished from one another.

In spite of the striking sequence conservation of these amplified fragments, without further analysis we cannot rule out the possibility that some derive from nonfunctional pseudogenes. However, in several cases evidence suggests

that the sequences reflect functional gene family members. Using these cloned PCR fragments as probes to screen libraries, nematode clones have now been isolated that have homology to the *Drosophila wg* gene extending well beyond the PCR fragment (G. Shackleford and J. Mason, personal communication). Likewise, in the case of human K^+ channels, the six classes of PCR clones have been used to isolate six different types of cDNA clone from human brain and spinal cord cDNA libraries. Three of these cDNA types have been partially sequenced, and all three exhibit homology to other K^+ channels well beyond the PCR segment (M. Ramaswami, M. Mathew, M. Gautam, and M. Tanouye, personal communication). Finally, one of the amplified tyrosine kinase fragments is identical in DNA sequence to the characterized *C. elegans abl* homolog. Although these results are encouraging, as with any such screen, proof that these or other conserved sequences derive from functional genes will require more extensive analysis.

Summary. PCR screening was used to identify and classify a wide variety of distinct sequence motifs, demonstrating its utility in the study of gene families. In addition, assuming that many of the conserved sequences derive from functional genes, these results may have considerable biological significance. First, the study indicates that *C. elegans* contains sequences closely related to a number of genes involved in *Drosophila* anteroposterior patterning. Given the genetic tractability of *C. elegans* and *Drosophila*, and the resolution with which developmental events can be observed, these organisms offer a unique opportunity to compare and contrast the ways in which similar genes are used to generate different body patterns. Similarly, these results suggest that a large family of tyrosine kinases exists in *C. elegans*. Thus, it may be possible to use genetic strategies to define pathways within which tyrosine kinases function to control cell growth and differentiation of individual cells. Finally, the identification of six K^+ channel sequences in human DNA suggests that multiple K^+ channel genes exist in humans. Studies of proteins encoded by these human sequences should reveal whether they may account for some of the functional diversity displayed by K^+ channels.

Note Added in Proof. Butler *et al.* (35) have reported the isolation of three additional K^+ channel homologs from *Drosophila* using conventional low-stringency hybridization.

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