Molecular evidence for the direct involvement of a protein kinase C in developmental and behavioural susceptibility to tumour-promoting phorbol esters in *Caenorhabditis elegans*

Yo TABUSE,* Tohru SANO,* Kiyoji NISHIWAKI* and Johji MIWA*†

* Fundamental Research Laboratories and † R & D Group, NEC Corporation, Tsukuba 305, Japan

The nematode *Caenorhabditis elegans* displays developmental and behavioural sensitivity to tumour-promoting phorbol esters. This sensitivity involves the gene *tpa-1*, which encodes two protein kinase C isoforms, TPA-1A and TPA-1B. Here we report the molecular nature of the sensitivity in this animal. Characterization of transposon Tc1-induced phorbol ester-resistant mutants has revealed that Tc1 was inserted in a region encoding the kinase domain, resulting in the loss of *tpa-1* products. Introduction of a genomic DNA containing the entire wild-type *tpa-1* locus into a Tc1-inserted mutant restored the sensitivity to tumour promoters, and *tpa-1* products were also produced. These results suggest that the function of wild-type TPA-1 is necessary and sufficient for tumour promoters to cause developmental and behavioural sensitivity in *C. elegans*.

INTRODUCTION

Tumour-promoting phorbol esters, such as phorbol 12-myristate 13-acetate [PMA; also known as 12-*O*-tetradecanoylphorbol 13-acetate (TPA)] and phorbol 12,13-didecanoate (PDD), have a variety of effects in many *in vivo* and *in vitro* biological and biochemical systems [1–4]. For example, cell division and differentiation, gene expression and cell morphology are all influenced by these compounds [1–3,5,6].

To understand the molecular mechanism by which phorbol esters produce these effects, we have adopted a genetic and developmental approach that takes advantage of the nematode *Caenorhabditis elegans*. *C. elegans* is a non-parasitic soil nematode which has many important advantages: it is readily amenable to general and molecular genetic manipulation, its development can be followed from cell to cell in exceptional detail, and each animal develops in an invariant manner. We have previously shown that PMA and PDD induce total developmental arrest and severely unco-ordinated behaviour in *C. elegans* [7,8]. By isolating and characterizing many PMA-resistant mutants, which behave normally and also develop and propagate in the presence of PMA, we have identified the gene *tpa-1* as the target of phorbol ester tumour promoters [9]. Molecular cloning has revealed that *tpa-1* encodes a homologue of protein kinase C (PKC), thus suggesting that a PKC molecule mediates the action of phorbol esters in modulating the development and behaviour of *C. elegans* [10].

PKC was initially isolated as a Ca^{2+}- and phospholipid-dependent diacylglycerol (DAG)-activated protein kinase [11,12]. It was later shown that tumour-promoting phorbol esters activate PKC by mimicking the endogenous activator DAG [13]. Since phorbol esters induce various physiological effects as mentioned above, PKC was implicated as a key enzyme in the regulation of diverse cellular functions via signal transduction pathways [14,15]. Molecular cloning studies have so far revealed that PKC consists of a large family of at least 11 isoforms, which can be divided into three major groups [16–18]: the first group comprising the Ca^{2+}-dependent 'conventional' PKCs (cPKCs), the second the Ca^{2+}-independent 'novel' PKCs (nPKCs), and the third other Ca^{2+}-independent 'atypical' PKCs (aPKCs), which are not activated by DAG or phorbol esters. The differential tissue distribution patterns exhibited by each isoform have led to the idea that distinct PKC isoforms may execute pivotal roles in separate signalling pathways, thus regulating a broad range of cellular functions as a PKC family. Although extensive *in vitro* studies have characterized the enzymic properties of PKC isoforms and described physiological responses elicited through their activation by phorbol esters, neither the molecular mechanism by which phorbol esters actually exert their effects *in vivo* nor a specific role for each isoform has as yet been established.

Our previous genetic analysis has suggested that the *tpa-1* gene product is a major, if not the only, functional target for the action of phorbol esters in *C. elegans* [9]. PKC isoforms other than TPA-1 (the product of gene *tpa-1*) have been reported recently, however, demonstrating that PKCs exist as heterogeneous molecular entities in *C. elegans* as well [19–21]. Also, we have shown recently that the *tpa-1* gene encodes two PKC isoforms, TPA-1A and TPA-1B [22]. Thus we asked the following questions: what is the molecular nature of PMA-resistant mutations, and is TPA-1 really a target that is necessary and sufficient for phorbol esters to induce developmental and behavioural distress in *C. elegans*? Answers to these questions should provide us with clues to understanding the underlying mechanisms of the phenotypes induced in *C. elegans* by phorbol esters and, consequently, the biological roles of PKCs and their associated molecules in various biological systems.

In this paper we first present, through characterization of transposon Tc1-induced PMA-resistant mutants, molecular evidence that a loss of susceptibility to phorbol esters in *C. elegans* is accompanied by the loss of the normal *tpa-1* product. Secondly, we show that introduction of the wild-type *tpa-1* gene into the mutant animal restores susceptibility to the agents. Both results

Abbreviations used: PMA, phorbol 12-myristate 13-acetate [also known as 12-*O*-tetradecanoylphorbol 13-acetate (TPA)]; PDD, phorbol 12,13-didecanoate; PKC, protein kinase C; DAG, diacylglycerol; cPKC, conventional PKC; aPKC, atypical PKC; nPKC, novel PKC; NGM, nematode growth medium; GST, glutathione S-transferase; Rol phenotype, roller phenotype conferred by marker plasmid pRF4.

together suggest that wild-type *tpa-1* gene function is necessary and sufficient for tumour-promoting phorbol esters to induce developmental and behavioural distress (i.e. susceptibility) in *C. elegans*.

From the present work, and previous genetic results suggesting that no loci other than *tpa-1* were found to be associated with the induced phenotypes [9], we propose that the *tpa-1* gene is a major and definitive component that directly mediates the action of tumour-promoting phorbol esters to cause distress in *C. elegans*. Other PKC isoforms may not be affected seriously by the compounds, if at all; otherwise, they may be involved in separate and parallel cellular signalling pathways, which do not lead to notable defects when the animal is treated with phorbol esters.

MATERIALS AND METHODS

C. elegans strains

C. elegans Bristol strain N2 was used as the standard wild type. *C. elegans* was cultured essentially as described [23]. The Tc1-induced PMA-resistant mutants MJ562 (*k529*) and MJ564 (*k531*) were isolated from the mutator strain NJ82, and MJ566 (*k532*) was isolated from RW7097, as described previously [10]. MJ563 (*k530*) was obtained by outcrossing the original isolate MJ562 (*k529*) to the wild-type (N2) and N2-derived marker strains [10].

Mutation site analysis

C. elegans genomic DNA was extracted from animals grown on agarose plates of nematode growth medium (NGM) containing 0.5 mM Ca^{2+} and seeded with *Escherichia coli* OP50 [23], as described previously [10]. To identify Tc1 insertion sites, we cloned the Tc1-containing 4 kb *Hin*dIII fragment (2.4 kb fragment plus 1.6 kb Tc1) from each of the three mutants into the *Hin*dIII site of the plasmid vector pUC18 [24] for the mutations *k529* and *k531* or pUC118 [25] for *k532*. Digestion of the plasmids with *Hin*dIII, as well as with *Eco*RV to remove Tc1 from the 4 kb fragment, divided the 4 kb fragment from each mutant into 1.8 and 0.6 kb bands, indicating that Tc1 insertion occurred at approximately the same position for each mutation. Each insertion site was determined by sequencing by the dideoxy chain termination method [26].

Preparation of antibodies to TPA-1

An antiserum to TPA-1 was prepared by standard procedures. The peptide CAFLNFSYTNPHFSK was synthesized, which corresponds to the C-terminal sequence of TPA-1 with the addition of an N-terminal cysteine residue to facilitate cross-linking to keyhole limpet haemocyanin. A Japan white rabbit was immunized with 0.5 mg of peptide that had been coupled to keyhole limpet haemocyanin with the use of maleimide. The animal was given three booster injections at 2-week intervals.

Production of glutathione S-transferase (GST)–TPA-1 polypeptide fusion proteins

The cDNA clone #1, cDNA1 [10], which encodes a 63.9 kDa polypeptide from Ile^{148} to the C-terminus of TPA-1A, was cloned into a *Bam*HI site of pGEX-2T [27] (Pharmacia LKB Biotechnology Inc.) with a *Bam*HI linker (Takara Syuzo Co.). This construction places cDNA1 downstream of and in-frame with the GST gene. cDNA1 was cut with *Ssp*I into three fragments of 0.6 kb, 0.4 kb and 0.7 kb (see Figure 1a). The 0.6 kb fragment (5′ end; cDNA1-a) encodes Ile^{148}–Asn^{358}, and the 0.7 kb fragment (3′ end; cDNA1-c) encodes Asn^{494} to the C-terminus. The former was cloned into a *Bam*HI site of pGEX-2T, and the latter was cloned into pGEX-3X that was cleaved with *Sma*I and *Eco*RI. The expression of each GST–TPA-1 fusion protein was driven by the isopropylthio-β-D-galactoside (IPTG)-inducible *tac* promoter. An overnight culture of *E. coli* strain transformed with each expression plasmid was diluted 1:100 into LB broth and cultured for 4 h at 37 °C. IPTG was added to a final concentration of 1 mM, and the incubation was continued for 2 h. Bacteria were harvested by centrifugation at 1800 ***g*** for 10 min, and the pellet from 10 ml of culture was suspended in 0.2 ml of lysis buffer containing 50 mM Tris/HCl (pH 8.0), 1 mM EDTA, 100 mM NaCl and 130 μM PMSF. The suspension was treated with lysozyme and DNase for 20 min at 25 °C and then centrifuged at 9000 ***g*** for 10 min. GST–TPA-1 fusion proteins were affinity-purified through glutathione–Sepharose 4B (Pharmacia LKB) and subjected to Western immunoblot analysis.

Protein isolation from *C. elegans* and immunoblot analysis

Nematodes grown on NGM were harvested by centrifugation and suspended in an equal volume of a disruption buffer containing 50 mM Tris/HCl (pH 8.0), 250 mM sucrose, 10 mM EGTA, 5 mM EDTA, 2 mM mercaptoethanol, 2 mM PMSF, 250 μg/ml leupeptin, 250 μg/ml pepstatin, 250 μg/ml trypsin inhibitor, 100 μg/ml antipain and 1 % aprotinin. The nematodes were ground in liquid nitrogen to a fine powder. After thawing, the samples were sonicated in three 10 s bursts with a Branson sonifier. The samples were then denatured at 95 °C for 5 min in Laemmli sample buffer [28], separated on a SDS/10 %-polyacrylamide gel (100 μg of protein per lane), and transferred electrophoretically to nitrocellulose membranes (Schleicher & Schüll). For immunoblot analysis, the nitrocellulose membranes were incubated sequentially with antiserum to TPA-1 (1:2000 dilution) and horseradish peroxidase-linked goat antibodies to rabbit immunoglobulin G (Organon Teknika Co.) (1:3000 dilution). Immune complexes were visualized using an enhanced chemiluminescence detection system (Amersham).

Generation of transgenic animals

MJ563 was transformed by injecting a mixture of the cosmid AA11 (50 μg/ml), which was provided by Dr. A. Coulson and Dr. J. Sulston (The Sanger Centre, Cambridge, U.K.) [29] and was shown to cover the entire *tpa-1* locus [22], and the marker plasmid pRF4 (100 μg/ml) into the gonads of gravid animals [30]. Transformants typically carried injected DNA as an extra-chromosomal array [30] and showed the roller (Rol) phenotype conferred by pRF4. F1 Rol progeny were cloned and allowed to self-fertilize. Clones that segregated Rol progeny in subsequent generations were selected as germline transgenic lines for further analysis. The frequency of Rol progeny in a brood of one Rol parent varied (from 20 to 60 %) among transgenic lines. Several Rol animals from each transgenic line were transferred on to an NGM plate and allowed to lay eggs, which were then transferred to a test NGM plate with or without PMA (1 μg/ml) and incubated for 3 days at 20 °C. Each plate was observed under a dissecting microscope and photographed with Tri-X film (Kodak).

RESULTS

Transposon insertion sites

The entire genomic region of the *tpa-1* gene, encompassing about 20 kb, was sequenced [22]. The *tpa-1* gene consists of 11 exons (Figure 1a), and two species of mRNA (2.8 kb and 2.4 kb) are transcribed from the locus. The 2.8 kb mRNA contains all 11

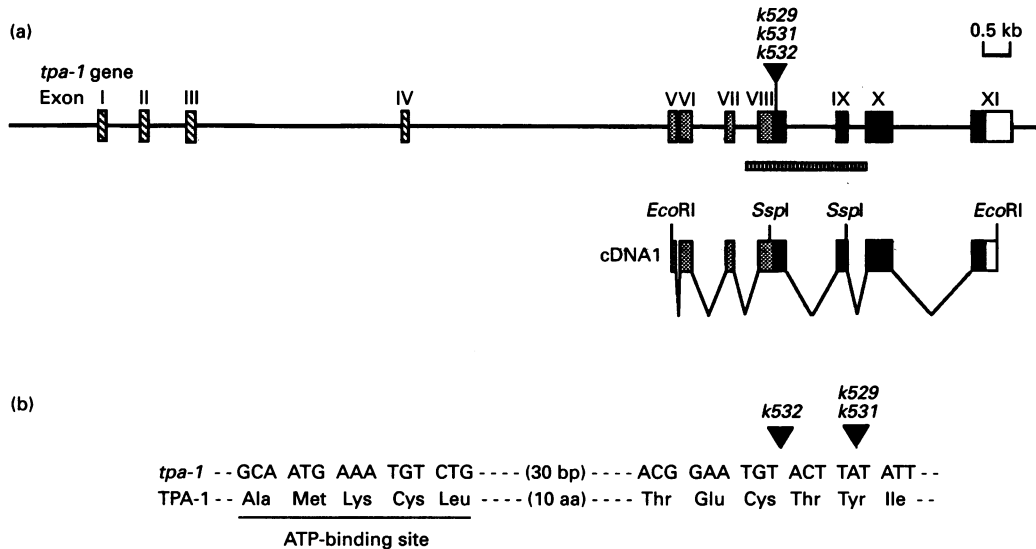

Figure 1 Physical map of the *tpa-1* locus and analysis of mutation sites

(**a**) The exon/intron organization of the *tpa-1* gene is shown [22]. Exons utilized only for TPA-1A are depicted as hatched boxes, those encoding part of the regulatory domain common to both TPA-1A and TPA-1B as stippled boxes, and those encoding the kinase domain as solid boxes. The open box represents the 3′ untranslated region. The locations of the three Tc1-induced mutations (▼) are indicated by their allelic names: *k529*, *k531* and *k532*. The restriction map of cDNA1, which is used to produce TPA-1–GST fusion proteins, is shown below. cDNA1 is cut with *Ssp*I into three fragments, of 0.6 kb (5′ end; cDNA1-a), 0.4 kb (cDNA1-b) and 0.7 kb (3′ end; cDNA1-c). The 2.4 kb *Hin*dIII fragment into which Tc1 was inserted is shown as a striped bar between the two physical maps. (**b**) Sequence of the Tc1 insertion sites. The exact Tc1 insertion site for each mutant was determined by sequencing the boundaries between Tc1 and *tpa-1*. The consensus sequence for an ATP-binding site is indicated by the solid line.

exons (exons I–XI), and predictably encodes a protein, TPA-1A, of 704 amino acids; the 2.4 kb mRNA, which contains exons V–XI, encodes another deduced protein, TPA-1B, of 567 amino acids. The predicted amino acid sequence of TPA-1B is identical to that of TPA-1A without the first 137 amino acids at the N-terminus. Both TPA-1A and TPA-1B are most similar to members of the mammalian Ca^{2+}-independent nPKC group [22].

MJ562 (*k529*), MJ564 (*k531*) and MJ566 (*k532*) are independently isolated Tc1-induced mutants that are resistant to tumour-promoting phorbol esters; these animals move normally and propagate in the presence of PMA and PDD. Molecular analysis of these mutants showed that, for each mutant, Tc1 was inserted in a 2.4 kb *Hin*dIII genomic fragment that contains exons encoding a small portion of the regulatory domain and about one-third of the kinase domain of TPA-1 (Figure 1a). Sequencing of the insertion-site regions revealed that, for these mutants, Tc1 was inserted in a region of exon VIII, shared in common by both TPA-1A and TPA-1B, about 40 bp downstream from a consensus sequence for the ATP-binding site in the kinase domain (Figure 1). Since in-frame stop codons appear within the inserted Tc1 [31], we infer that the mutations cause premature termination of TPA-1 about 40 amino acids downstream of the position corresponding to the 5′ end of the Tc1 insertion for both *k529* and *k531*, and about 30 amino acids downstream for *k532*. Since Tc1 is inserted in exon VIII, all the mutations analysed here should have resulted in the simultaneous loss of wild-type TPA-1A and TPA-1B.

TPA-1A and TPA-1B in phorbol ester-resistant mutants

To investigate the effects of the Tc1 insertion in the mutants on TPA-1 expression, we generated a rabbit polyclonal antibody to a short synthetic peptide corresponding to a C-terminal sequence of TPA-1. This peptide was chosen because it displays no identity to any other previously characterized PKC molecules.

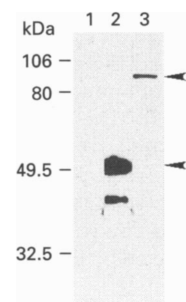

Figure 2 Specificity of the anti-TPA-1 antibody

Each TPA-1 segment encoded by cDNA1-a (lane 1), cDNA1-c (lane 2) and cDNA1 (lane 3) was expressed separately in *E. coli* as a fusion protein with GST. Although cDNA1-c and cDNA1 encode the C-terminus of TPA-1 proteins, cDNA1-a does not. Immunoblot analysis of cell extracts was performed as described in the Materials and methods section. Arrowheads indicate protein bands of apparent molecular mass 50 kDa (lane 2) and 90 kDa (lane 3) which were detected by the antibody. A unidentified band of 40 kDa, which may have resulted from degradation, was also observed in lane 2.

The specificity of the antibody was assessed in Western immunoblot analysis of various TPA-1 segments that we obtained by expressing cDNA1 [10], as well as its *Eco*RI/*Ssp*I fragments (Figure 1), in *E. coli* as a fusion protein with GST. As shown in Figure 2, the antibody successfully recognized TPA-1 segments encoded by cDNA1 and cDNA1-c, both of which encode the C-terminus of TPA-1. On the other hand, no bands were detected in the extract from *E. coli* expressing 0.6 kb cDNA-a, which does not encode the C-terminus of TPA-1. The calculated molecular mass of GST is 26 kDa, and those of the TPA-1 segments encoded by cDNA1 and cDNA1-c are 64 kDa and 24 kDa respectively. Therefore the apparent molecular masses of the

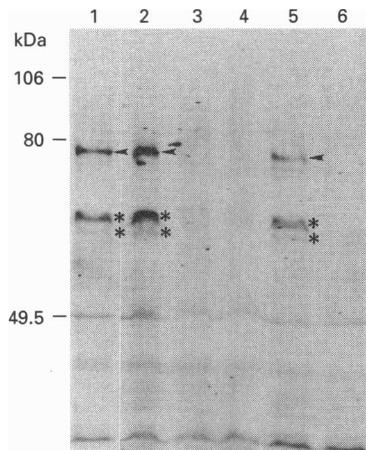

Figure 3 Immunoblot analysis of TPA-1 in PMA-resistant mutants

Protein extracts were prepared from the following strains: lane 1, wild-type N2; lane 2, NJ82; lane 3, MJ562; lane 4, MJ564; lane 5, RW7097; lane 6, MJ566. A protein band with an apparent molecular mass of 76 kDa, indicated by an arrowhead (lanes 1, 2 and 5), corresponds to TPA-1A. Asterisks indicate 60 kDa and 58 kDa bands, which correspond to TPA-1B. Several unidentified bands that cross-reacted with the anti-TPA-1 antibody are visible in all of the samples. Molecular mass markers are indicated on the left.

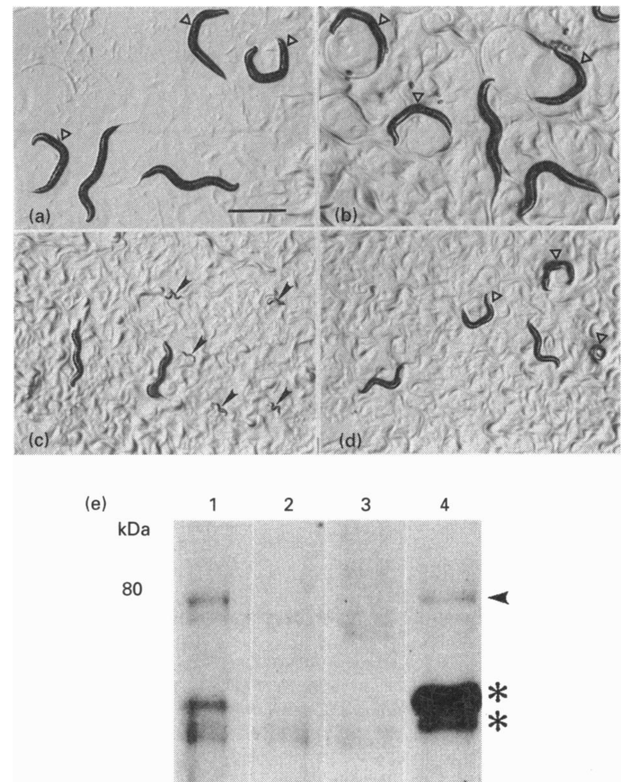

Figure 4 Phenotypic characterization of lines transformed with cosmid AA11

The growth of transgenic animals in the absence or presence of PMA is shown. Embryos from line #6-4, derived by transformation of the PMA-resistant strain MJ563 (*k530*) with the cosmid AA11 and the marker plasmid pRF4 (**a** and **c**), and from line #3-a, derived from transformation of MJ563 (*k530*) with pRF4 alone (**b** and **d**), were incubated in the absence (**a** and **b**) or presence (**c** and **d**) of PMA (1 μg/ml). Arrowheads indicate animals whose growth was arrested by PMA at the first (L1) or the second (L2) larval stage; open triangles indicate animals showing the C-shaped Rol phenotype. Although animals resistant to phorbol esters grew more slowly and to a smaller size in the presence of PMA (**b** and **d**) than in its absence (**a** and **c**), they reproduced well in the presence of PMA. Scale bar, 0.5 mm. (**e**) Immunoblot analysis of TPA-1 in transgenic animals. Protein extracts from N2 (lane 1), MJ563 (lane 2), transformant #3-a (lane 3) and transformant #6-4 (lane 4) were subjected to immunoblot analysis. The arrowhead indicates the 76 kDa TPA-1A band, and asterisks indicate the 60 and 58 kDa TPA-1B bands.

detected bands, 90 kDa for cDNA1 expression and 50 kDa for cDNA1-c, are in good agreement with those expected for the fusion proteins.

Immunoblot analysis with the anti-TPA-1 antibody specifically detected 76 kDa, 60 kDa and 58 kDa bands in extracts prepared from the wild-type (N2) and the parental (NJ82 and RW7097) strains of the PMA-resistant mutants. The 60 kDa band was more abundant than the 58 kDa band. These bands were not detected in extracts prepared from the Tc1-induced mutants (Figure 3). The antibody, when preincubated with the antigen peptide prior to immunoblot failed to detect any of the three bands (results not shown). The result further supports the idea that these three bands detected by the antibody correspond to the products of *tpa-1*.

Since the calculated molecular mass of TPA-1A is 80 kDa, the band with an apparent molecular mass of 76 kDa corresponds well to TPA-1A. Both the 60 kDa and 58 kDa bands are good candidates for TPA-1B, with a predicted molecular mass of 65 kDa. We suggest that both represent TPA-1B, since some post-translational modification, such as phosphorylation of TPA-1B, could result in double bands as reported for rat PKC-ϵ [32]. This suggestion is supported by two observations: (1) transgenic animals transformed with a genomic DNA clone, the cosmid AA11, containing the entire *tpa-1* region produced both bands (see Figure 4e); and (2) structural analysis of *tpa-1* mRNAs detected no apparent size differences in the TPA-1B coding region [22].

Restoration of wild-type sensitivity to PMA in transgenic resistant mutants

The results described above suggested that loss of TPA-1 in the mutants was responsible for their resistance to PMA. If this were the case, the introduction and functional expression of the wild-type *tpa-1* gene in PMA-resistant mutants should restore TPA-1 activity and hence sensitivity to PMA. To examine this possibility, we microinjected the cosmid AA11 [29], which includes the entire *tpa-1* locus, into the gonads of MJ563 (*k530*). MJ563 is a PMA-resistant mutant strain obtained by out-crossing the Tc1-induced mutant MJ562 (*k529*) ten times to the wild-type N2 and N2-derived marker strains. The transformation marker plasmid pRF4, which carries the dominant *rol-6* (*su1006*) allele [33] that confers a Rol phenotype, was used as a selectable co-segregation marker for the injected *tpa-1* gene.

Several lines of germline transformants that reproducibly gave rise to animals with the Rol phenotype were isolated, and each line was examined for growth in the presence of PMA. In the absence of PMA about 40 % of the fully grown progeny of the transformant line #6-4 expressed the Rol phenotype (Figure 4a and Table 1). In the presence of PMA, however, only non-Rol segregants grew to be adults. In addition, many small growth-arrested larvae were observed on PMA-containing plates (Figure 4c). The control line #3-a, transformed with pRF4 alone, produced full-grown Rol animals in the presence of PMA (Figure 4d). It remains to be confirmed whether the growth-arrested larvae displayed PMA-sensitive behaviour. We assume that they did, however, because the Rol animals with and without apparent

Table 1 Phenotypic characterization of germline transformants

Embryos were incubated in the absence or prsence of PMA (1 μg/ml) as described in the Materials and methods section, and the animals were subsequently scored according to the following categories: Rol, roller animals grown larger than fourth-stage larvae (L4); non-Rol, non-roller animals grown larger than L4 larvae; arrested, growth-arrested animals at the L1 or L2 stage; missing or unhatched, animals missing after incubation and unhatched embryos.

	−PMA				+PMA				
						Grown			
Line	No. of embryos tested	Rol	non-Rol	Missing or unhatched	No. of embryos tested	Rol	non-Rol	Arrested	Missing or unhatched
#6-4	110	39	53	18	112	0	52	47	13
#3-a	80	43	33	4	80	34	36	3	7

superimposition of PMA-induced sensitive behaviour were distinguishable.

We also detected the 76 kDa, 60 kDa and 58 kDa protein bands in extracts of the transformant line #6-4 by immunoblot analysis (Figure 4e, lane 4). Thus introduction of the wild-type *tpa-1* gene restored not only sensitivity to PMA but also the expression of both TPA-1A and TPA-1B in the PMA-resistant mutant MJ563.

DISCUSSION

Biochemical studies have indicated that tumour-promoting phorbol esters such as PMA and PDD, by mimicking the second messenger DAG, elicit a variety of biological effects through activation of PKC in cellular signalling pathways [34–36]. However, evidence that directly connects phorbol ester-induced biological effects to PKC activation *in vivo* is limited. As a result of the isolation of phorbol ester-resistant mutants of *C. elegans*, we have previously provided genetic and molecular evidence that a homologue of PKC, encoded by the gene *tpa-1*, mediates the phorbol ester-induced developmental and behavioural defects in *C. elegans* [9,10]. Our recent structural analysis of the *tpa-1* gene has indicated that it encodes two PKC isoforms, TPA-1A and TPA-1B, which are most similar to mammalian members of the Ca^{2+}-independent nPKC group [22]. TPA-1A and TPA-1B differ only in their N-terminal region; the latter lacks the first 137 N-terminal amino acids found in the former.

In this paper we have described the three mutations *k529*, *k531* and *k532* induced by the *C. elegans* transposon Tc1, which confer PMA resistance on *C. elegans*. Each mutation was shown to result from insertion of Tc1 into exon VIII of *tpa-1*, which encodes a part of the kinase domain common to both TPA-1A and TPA-1B. These insertions would result in the production of incomplete TPA-1 proteins, lacking about 80% of the kinase domain. Indeed, immunoblot analysis of extracts from the mutants failed to detect bands corresponding to TPA-1A and TPA-1B. This result suggests that the mutants lack full-length, functional TPA-1 proteins. Thus we propose that the mutants have acquired resistance to or lost sensitivity to phorbol esters by a loss of the *tpa-1* products.

This idea is further supported by transformation experiments in which the wild-type *tpa-1* gene was introduced into the mutant MJ563 (*k530*). Transgenic animals transformed with the wild-type *tpa-1* gene were sensitive to phorbol esters and produced 76 kDa, 60 kDa and 58 kDa proteins that react with the antibody to the C-terminal peptide of TPA-1. The results presented here indicate that function of the wild-type *tpa-1* gene is necessary and sufficient for phorbol esters to cause severe behavioural and developmental defects in *C. elegans*.

It has been reported that overexpression of kinase-negative or truncated mutant genes of PKC isoforms or Raf-1 kinase, which were introduced ectopically into cells, exerts dominant negative effects on their wild-type counterparts, thus interrupting cellular signalling mediated by these kinases [37–40]. We did not clarify the presence of the predicted truncated products in the phorbol ester-resistant mutants, since in the present study we used an antibody specific to the C-terminal peptide of TPA-1. The following observations, however, led us to think it unlikely that the truncated *tpa-1* products cause dominant negative effects to induce phorbol ester-resistant phenotypes in our mutants. (1) The previous genetic analysis [9] and the present work indicate that the *tpa-1* gene is the sole major constituent mediating the action of phorbol esters in *C. elegans*. (2) The expression of mutant genes, which we have analysed here, is driven by the same intrinsic promoter that functions in the wild-type gene. (3) We have detected no sign of overexpression of *tpa-1* mRNA in the mutant (results not shown). (4) An antibody that we raised against an N-terminal sequence unique to TPA-1A did not detect any bands corresponding to the predicted truncated product in immunoblot analysis (results not shown), although the presence of truncated TPA-1B remains to be examined.

Previous reports have shown that *C. elegans* also possesses a PKC family containing various isoforms [19–21]. Our present results indicate that *tpa-1*, which encodes PKC-δ and θ homologues [41,42] among the PKC isoforms, is one definitive cellular component that mediates the disastrous consequences of tumour-promoting phorbol esters in *C. elegans*.

Although the Tc1 insertion mutants lacked the normal *tpa-1* gene products, none showed any apparent growth or behavioural abnormalities in the absence of tumour promoters [9]. Several interpretations could explain this observation. (1) TPA-1 has no essential role in *C. elegans*. Although functional, it is usually 'silent and sleeping'. When 'awakened' (activated) by agents such as phorbol esters, it acts detrimentally against the animal. (2) It is dispensable for development and behaviour, but has some other important functions that we cannot easily observe. Developmental and behavioural disturbances thus do not reflect its regular function. (3) The mutants produce truncated products with residual TPA-1 activity. (4) It has an important role for development and behaviour, but functions as only one member of a multiple gene family that redundantly execute similar roles essential for survival. At present, no conclusive evidence is available to support one interpretation over the others. However, it is empirically sound that the affected phenotypes reflect at least some functional aspects of the gene concerned and its product. This eliminates the first and second interpretations, because both assume that the phenotypes affected by the phorbol agents have nothing to do with the regular, normal function of TPA-1.

The third interpretation assumes that the truncated products, presumably with the entire sequence deleted downstream from the ATP-binding site, perform apparently normal functions in the absence of phorbol esters as do the wild-types. However, we wish to consider the fourth interpretation because, consistent with this, multiple PKC isoforms have been reported as described above [19–21]. Also, we observed essentially the same PKC activity and phorbol ester binding activity in the mutant MJ563 (T. Sassa, Y. Tabuse and J. Miwa, unpublished work) as in the wild-type animal [19]. If we assume that *tpa-1* is a member of a *C. elegans* PKC family and that its function is indispensable for survival, then other members should encode proteins which are either less sensitive or inaccessible to tumour promoters. In this respect, aPKCs, for instance, which are not activated by either DAG or phorbol esters [17], might be candidates for such family members.

The *unc-13* gene of *C. elegans* has been reported to encode a protein that contains a sequence that is highly similar to a cysteine-rich and presumptive phorbol-binding region of PKC [43]. Furthermore, Ahmed et al. [44] showed that the cysteine-rich region of the *unc-13* product (UNC-13) expressed in *E. coli* binds to a tumour-promoting phorbol ester, phorbol 12,13-dibutyrate. Given that *tpa-1* mutants are apparently homozygous for the wild-type *unc-13* allele and yet are resistant to phorbol esters, then either UNC-13 might be inaccessible to phorbol esters *in vivo* or, alternatively, phorbol ester binding to UNC-13 may have little effect on its function in behaviour and development.

Long-term exposure of cells to phorbol esters down-regulates PKC [45,46]. PKC exists as a family of multiple isoforms [15,17,18], and many isoforms are reported to be down-regulated in response to phorbol esters [47–49]. However, the biological significance of PKC down-regulation, and especially whether it has any relevance to phorbol ester-induced biological effects, is not well understood. The effects of phorbol esters on *C. elegans* remain prominent during continuous exposure for several days [8]. The lasting expression of these defects under continuous treatment with phorbol esters, together with their suppression by loss of the *tpa-1* proteins, suggests that phorbol esters do not induce TPA-1 depletion in *C. elegans*. Rather, these results suggest that sustained or excessive activation of TPA-1 is responsible for the observed effects of phorbol esters in *C. elegans*.

We thus have a whole-animal (i.e. *in vivo*) assay system to hand with which to assess the activities of various PKC isoforms. Further analysis, e.g. by expressing TPA-1A, TPA-1B or other PKC isoforms under the *tpa-1* promoter, would give us a better insight into the biological meaning of differential tissue and developmental (spatio-temporal patterns of) expressions of each PKC isoform.

How does a signal transmitted by TPA-1 relate to the development and behaviour of *C. elegans*? More generally, how does a protein encoded by a member of a multigene family achieve its mission of sending a specific signal to specific targets without interference from related proteins and other targets? The amenability of *C. elegans* to powerful molecular genetic analysis should make this nematode an excellent system for the genetic dissection of cellular signalling pathways and, more generally, of biologial information processing.

We thank Alan Coulson and John Sulston for physical mapping and cosmid clones; Jim Kramer for the plasmid pRF4; Ed Hedgecock for NJ82; Robert Waterston and Ikue Mori for RW7097; Victor Ambros, Diane Levitan and Ikue Mori for the microinjection technique; Toshie Miyamoto for assistance in experiments; and S. Bernice Miwa for critical reading of the manuscript.

Received 25 February 1995/29 June 1995; accepted 12 July 1995

REFERENCES

1 Rovera, G., O'Brien, T. G. and Diamond, L. (1977) Proc. Natl. Acad. Sci. U.S.A. **74**, 2894–2898
2 Bresch, H. and Arendt, U. (1978) Naturwissenschaften **65**, 660–662
3 Otte, A. P., Koster, C. H., Snoek, G. T. and Durston, A. J. (1988) Nature (London) **334**, 618–620
4 Minke, B., Rubinstein, C. T., Sahly, I., Timberg, R. and Selinger, Z. (1990) Proc. Natl. Acad. Sci. U.S.A. **87**, 113–117
5 Choi, P. M., Tchou-Wong, K. and Weinstein, I. B. (1990) Mol. Cell. Biol. **10**, 4650–4657
6 Chao, Y. and Pellegrini, M. (1993) Mol. Cell. Biol. **13**, 934–941
7 Miwa, J., Tabuse, Y., Nishiwaki, K., Yamasaki, H. and Furusawa, M. (1980) Igaku no Ayumi **114**, 910–912
8 Miwa, J., Tabuse, Y., Yamasaki, H. and Furusawa, M. (1982) Cancer Res. Clin. Oncol. **104**, 81–87
9 Tabuse, Y. and Miwa, J. (1983) Carcinogenesis **4**, 783–786
10 Tabuse, Y., Nishiwaki, K. and Miwa, J. (1989) Science **243**, 1713–1716
11 Takai, Y., Kishimoto, A., Inoue, M. and Nishizuka, Y. (1977) J. Biol. Chem. **252**, 7603–7609
12 Takai, Y., Kishimoto, A., Iwasa, Y., Kawahara, Y., Mori, T. and Nishizuka, Y. (1979) J. Biol. Chem. **254**, 3692–3695
13 Castagna, M., Takai, Y., Kaibuchi, K., Sano, K., Kikkawa, U. and Nishizuka, Y. (1982) J. Biol. Chem. **257**, 7847–7851
14 Nishizuka, Y. (1986) Science **233**, 305–312
15 Nishizuka, Y. (1988) Nature (London) **334**, 661–665
16 Ohno, S., Akita, Y., Hata, A., et al. (1991) Adv. Enzyme Regul. **31**, 287–303
17 Hug, H. and Sarre, T. F. (1993) Biochem. J. **291**, 329–343
18 Wilkinson, S. E. and Hallam, T. J. (1994) Trends Pharmacol Sci. **15**, 53–57
19 Sassa, T. and Miwa, J. (1992) Biochem. J. **282**, 219–223
20 Land, M., Islas-Trejo, A., Freedman, H. and Rubin, C. S. (1994) J. Biol. Chem. **269**, 9234–9244
21 Land, M., Islas-Trejo, A. and Rubin, C. S. (1994) J. Biol. Chem. **269**, 14820–14827
22 Sano, T., Tabuse, Y., Nishiwaki, K. and Miwa, J. (1995) J. Mol. Biol. **251**, 477–485
23 Brenner, S. (1974) Genetics **77**, 71–93
24 Yanisch-Perron, C., Vieira, J. and Messing, J. (1983) Gene **33**, 103–119
25 Vieira, J. and Messing, J. (1987) Methods Enzymol. **153**, 3–11
26 Sanger, F., Nicklen, S. and Coulson, A. (1977) Proc. Natl. Acad. Sci. U.S.A. **74**, 5463–5467
27 Smith, D. B. and Johnson, K. S. (1988) Gene **67**, 31–40
28 Laemmli, U. K. (1970) Nature (London) **227**, 680–685
29 Coulson, A., Sulston, J., Brenner, S. and Karn, J. (1986) Proc. Natl. Acad. Sci. U.S.A. **83**, 7821–7825
30 Mello, C. C., Kramer, J. M., Stinchcomb, D. and Ambros, V. (1991) EMBO J. **10**, 3959–3970
31 Rosenzweig, B., Liao, L. W. and Hirsh, D. (1983) Nucleic Acids Res. **11**, 4201–4209
32 Koide, H., Ogita, K., Kikkawa, U. and Nishizuka, Y. (1992) Proc. Natl. Acad. Sci. U.S.A. **89**, 1149–1153
33 Kramer, J. M., French, R. P., Park, E. and Johnson, J. J. (1990) Mol. Cell. Biol. **10**, 2081–2090
34 Cantrell, D. A., Davies, A. A. and Crumpton, M. J. (1985) Proc. Natl. Acad. Sci. U.S.A. **82**, 8158–8162
35 Orellana, S. A., Solski, P. A. and Brown, J. H. (1985) J. Biol. Chem. **260**, 5236–5239
36 Helper, J. R., Earp, H. S. and Harden, T. K. (1988) J. Biol. Chem. **263**, 7610–7619
37 Kolch, W., Heidecker, G., Lloyd, P. and Rapp, U. R. (1991) Nature (London) **349**, 426–428
38 MacNicol, A. M., Muslin, A. J. and Williams, L. T. (1993) Cell **73**, 571–583
39 Berra, E., Diaz-Meco, M. T., Dominguez, I., Municio, M. M., Sanz, L., Lozano, J., Chapkin, R. S. and Moscat, J. (1993) Cell **74**, 555–563
40 Hirai, S., Izumi, Y., Higa, K., et al. (1994) EMBO J. **13**, 2331–2340
41 Ono, Y., Fujii, T., Ogita, K., Kikkawa, U., Igarashi, K. and Nishizuka, Y. (1988) J. Biol. Chem. **263**, 6927–6932
42 Osada, S.-I., Mizuno, K., Saido, T. C., Suzuki, K., Kuroki, T. and Ohno, S. (1992) Mol. Cell. Biol. **12**, 3930–3938
43 Maruyama, I. N. and Brenner, S. (1991) Proc. Natl. Acad. Sci. U.S.A. **88**, 5729–5733
44 Ahmed, S., Maruyama, I. N., Kozma, R., Lee, J., Brenner, S. and Lim, L. (1992) Biochem. J. **287**, 995–999
45 Ballester, R. and Rosen, O. M. (1985) J. Biol. Chem. **260**, 15194–15199
46 Lindner, D., Gschwendt, M. and Marks, F. (1991) Biochem. Biophys. Res. Commun. **176**, 1227–1231
47 Kariya, K. and Takai, Y. (1987) FEBS Lett. **219**, 119–124
48 Akita, Y., Ohno, S., Yajima, Y. and Suzuki, K. (1990) Biochem. Biophys. Res. Commun. **172**, 184–189
49 Liyanage, M., Frith, D., Livneh, E. and Stabel, S. (1992) Biochem. J. **283**, 781–787