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Molecular cloning and expression pattern of *rpr-1*, a resiniferatoxin-binding, phosphotriesterase-related protein, expressed in rat kidney tubules

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Abstract Bacterial phosphotriesterases are enzymes that hydrolyse phosphotriester-containing organophosphate pesticides. Resiniferatoxin is a vanilloid that desensitises nociceptive neurons. By screening a rat cDNA library with labelled resiniferatoxin, we unexpectedly isolated a novel rat phosphotriesterase homologue, here named *rpr-1*, that encodes a 349 amino acid, 39 kDa protein (confirmed by in vitro translation). Northern blotting and in situ hybridisation show expression primarily in proximal tubules of the kidney, in which *rpr-1* distribution correlates with resiniferatoxin-binding activity. These results suggest an unsuspected link between the phosphotriesterase enzyme family and resiniferatoxin toxicity and pharmacology.

Key words: Resiniferatoxin; Phosphotriesterase; Kidney; Rat

1. Introduction

Microbial phosphotriesterases are a group of zinc metalloenzymes that catalyse the hydrolysis of a range of phosphotriester compounds including organophosphate insecticides and nerve gases, for example *parathion, paraoxon, sarin* and *soman.* They have attracted much interest in recent years because of their promise as agents for detoxification of organophosphate-contaminated industrial and agricultural waste [1] and because of their ability to protect against attack by organophosphate neurotoxins [2]. They have therefore been studied closely at the biochemical, molecular genetic and crystallographic levels [3–7].

So far, all proteins with proven phosphotriesterase activity have been isolated from prokaryotic sources, but recently Hou et al. (1996) have isolated from mice a cDNA sequence with significant homology to *Flavobacterium spp* parathion hydrolase and the phosphotriesterases of *Escherichia coli* and *Pseudomonas diminuta,* particularly in the regions of the enzyme's active sites. The functions and possible enzyme activities of the protein encoded by this mouse gene, *mpr56-1,* have not yet been determined.

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Abbreviations: RTX, resiniferatoxin; RTX-PAL, resiniferanol-9,13,14-orthophenylacetate-20-(3-azido, 4 methoxyphenyl) acetate

The sequence of *rpr-1* has the EMBL accession number X99477.

We have been studying clones from a rat cDNA expression library that have been selected for their ability to bind resiniferatoxin, a plant-derived vanilloid that desensitises certain nociceptive neurons and is therefore of great pharmacological interest [9–11]. The cDNAs of most of these resiniferatoxin-binding clones represent mRNAs that are expressed in the nervous system [12,13]. One, however, was found to be expressed primarily in the kidney. Here, we report the sequence and expression pattern of this cDNA, *rpr-1*. Our results suggest that *rpr-1* is a resiniferatoxin-binding rat homologue of prokaryotic phosphotriesterases that is expressed primarily in proximal renal tubules.

2. Materials and methods

2.1. Isolation of Clone rpr-1

The cone containing *rpr-1*, among others, was obtained by screening of a rat dorsal root ganglion (DRG) cDNA library with resiniferanol-9,13,14-orthophenylacetate-20- (3-azido, 4 methoxyphenyl) acetate (RTX-PAL [13]). A neonatal rat cDNA expression library was plated at 5×10^4 pfu/150 mm plate and after incubation for 3.5 h at 42°C the plates were overlaid with Hibond-C membranes (Amersham) impregnated with 10 mM IPTG for induction of protein expression, and incubated for a further 4 h at 37°C. Filters were incubated in 10 nM RTX-PAL solution in PBS for 30 min at room temperature, washed briefly in PBS and UV-irradiated for 90 s. The filters were then washed in PBS, blocked with 5% haemoglobin (Sigma) and incubated overnight at 4°C with rabbit polyclonal anti-RTX serum. After 3 washes in PBS, 50 mM Tris (pH 7.4) and finally 50 mM Tris (pH 9.5), they were incubated for 15 min with Nitroblue tetrazolium and bromochloroindolyl phosphate (Sigma) in Tris (pH 9.5), 5 mM $MgCl_2$, 50 mM NaCl. After enzymatic development for 5–30 min, filters were washed in PBS and dried. Control filters were treated identically but not UV irradiated. Positive clones were rescued into pBluescript II plasmids. After screening 300 000 pfu from an unamplified library, only one full-length clone corresponding to *rpr-1* was identified.

2.2. Sequence analysis

Sequencing of the *rpr-1* cDNA was carried out using *Taq* 'Dye-deoxy'™ terminator chemistry on an ABI 373A automated sequencer. Initial sequencing primers were complementary to the T3 and T7 sites on the *pBluescript* Vector, and subsequent ones were designed to match newly acquired sequence. Each part of the sequence was read at least once on each strand. Comparison of the sequences with the EMBL database was performed using the E-mail FASTA service of EBI (FASTA@ebi.ac.uk), while alignment and translation-by-computer were performed using the SEQAID program, obtained from netserv@ebi.ac.uk.

2.3. In vitro translation

Coupled in vitro transcription and translation of *rpr-1* cDNA was carried out in vitro using Promega's TNT™ reticulocyte lysate system, using biotinylated leucine (tRNAnscend™, also from Promega) to label the peptides produced. Plasmids carrying luciferase cDNA

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were used as positive controls for translation reactions. Translation products were analysed on 10% SDS-PAGE gels [14].

2.4. Expression studies

Northern blotting was carried out by separating polyA+ RNA from neonatal rat tissues (2 μg/track) on a 1.2% agarose/formaldehyde gel and blotting it on to a HiBond N membrane (Amersham International). RNA was fixed to the membrane by UV irradiation ($\lambda = 254$ nm, 3 min) and baking dry at 80°C for 60 min. Non-specific binding sites were blocked with 50% formamide, 5× SSC, 5× Denhardts' solution, 100 μg/ml salmon sperm DNA, 50 ng/ml yeast tRNA, 0.1% SDS, then the blot was hybridised with 3×10^6 cpm of denatured *rpr-1* (radiolabelled by random priming) in the same solution at 42°C for 40 h. After hybridisation, the filters were washed at room temperature in 2× SSC (20 min) then in 2× SSC 0.2% SDS, then in 0.2% SSC 0.2% SDS at 65°C for 20 min. The washed filters were then exposed to Kodak XAR-5 film at −70°C overnight. After stripping, filters were rehybridised with a probe for the house-keeping gene *L27* to confirm that tracks contained an equal amount of RNA.

In situ hybridisation was carried out using Amersham's 'RNA Colour Kit', but with some departures from the standard protocol provided with it. The T7 and T3 promoters were used to make fluorescein-labelled sense and antisense probes from linearised RTX-10b cDNA, according to the protocol provided with the kit. Sprague-Dawley rat kidneys were fixed in 10% formalin, embedded in wax and sectioned using standard histological procedures. They were then dewaxed, incubated in 0.2 M HCl for 10 min at room temperature, washed in 0.1% Triton X-100 in PBS for 1 min, rinsed in PBS and treated with 10 μg/ml proteinase K in PBS for 10 min at room temperature. They were then allowed to dry. Prior to hybridisation, sections were incubated for 1 h in the hybridisation mix supplied with the RNA Colour Kit, then hybridised with the fluorescein-labelled probes in hybridisation solution at 53°C overnight. The following day, they were washed in 2× SSC (2× 5 min, room temperature), in 0.2× SSC 0.1% SDS at 53°C for 10 min, rinsed in 2× SSC then incubated in a 1% solution of blocking agent (from the kit) for 1 h. Sheep antifluorescein primary antibody was diluted 1:100 in PBS and applied to the sections overnight at 4°C. Slides were then washed in PBS, and a 1:500 solution of mouse anti-alkaline phosphatase was applied to them for 30 min. They were again washed in PBS, and incubated in a 1:50 solution of anti-mouse IgG labelled with 10 nm gold particles for a further 30 min. The sections were then washed again in PBS, post-fixed in 10% formalin for 1 h, and stained with haematoxylin and eosin and viewed under dark-field optics.

2.5. ^{3}H-RTX binding to kidney sections

Sections from the same blocks used for in situ hybridisation studies were dewaxed in xylene, rehydrated through graded alcohols and incubated for 1 h in incubation medium (PBS containing 0.02% Triton X-100 and 1 mg/ml ZnI_2). The liquid was then shaken off the slides, and approximately 50 μl fresh incubation medium containing ^{3}H-RTX (40 μCi/ml) was placed on to the sections and incubated on them for 3 h at room temperature in a humidified chamber. Slides were washed in three changes of approximately 100 ml of incubation buffer, dried, coated with LM-1 nuclear emulsion (Amersham) and exposed for 7 days at 4°C. They were viewed on a Zeiss photomicroscope under dark-field illumination. Slides that had not been incubated in ^{3}H-RTX were used as controls for non-specific silver fixation or photoreflective effects.

3. Results

3.1. Sequence of rpr-1

Clone *rpr-1* was isolated from a DRG cDNA expression library on the basis of its ability to bind resiniferatoxin [13]. The base sequence of clone 10b cDNA, determined by *Taq* Dye-deoxy™ chemistry, may be found in the EMBL database (accession number X99477). The 1728 base sequence contains an open reading frame of 1047 bases, commencing with an ATG codon at position 109, within a good Kozak consensus [15], and ending with a TAG termination codon at position 1156. A consensus poly-adenylation signal, AATAAA, appears downstream of the termination codon at position 1702, and a short length of poly-A may be seen at the extreme 3′ end of the cloned cDNA.

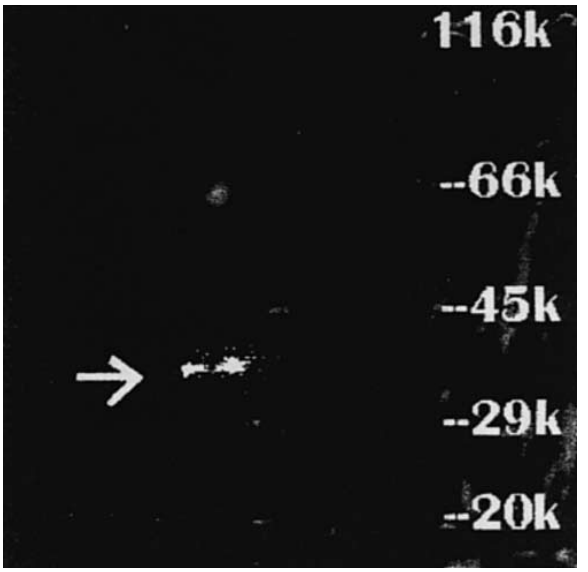

Fig. 1. In vitro translation of *rpr-1* cDNA: a single band appears at the approximate predicted molecular mass of *rpr-1* (arrow).

The only open reading frame of *rpr-1* yields a 349 amino acid, 39 kDa peptide with no secretory leader sequence and no strongly hydrophobic (potentially membrane-spanning) stretches: it is therefore presumably cytoplasmic. To confirm the size of the predicted protein, the clone was transcribed and translated in an in vitro assay using the TNT coupled reticulocyte lysate system. Only one translation product (≈39 kDa) was observed (Fig. 1).

Comparison of the *rpr-1* cDNA with the contents of the EMBL and Genbank databases, using the FASTA algorithm, reveals a close similarity (90.8% identity at the nucleic acid level) to the recently reported murine gene, *mpr56-1* [8]. The similarity between these two genes is even higher (92%) at the amino acid level (Fig. 2), and the proteins from the two species are exactly the same length (349 amino acids). In particular, the four histidine residues (H^{26}, H^{28}, H^{201}, H^{230}) and the aspartate (D^{298}) which co-ordinate with metal ions and are absolutely necessary for bacterial phosphotriesterase function [6,16], are conserved (Fig. 2). These data argue strongly that *rpr-1* is the rat homologue of the mouse phosphotriesterase-related gene *mpr56-1*. We therefore propose that it should be named *rpr-1* (for 'rat phosphotriesterase-related gene 1', following the naming convention used by Hou et al. for their mouse phosphotriesterase-related gene, *mpr56-1*).

3.2. Expression of rpr-1

The expression pattern of *rpr-1* was assessed by Northern blotting of tissues from new-born rats (Fig. 3). A single transcript, migrating at approximately 1.8 kb, was observed and confirmed that the cloned Kid10b/*rpr-1* cDNA was essentially complete. Of the organs studied (CNS, spinal ganglia, adrenal gland, thymus, liver, kidney, heart, lung, muscle and skin) only kidney produced a strong signal, with very weak signals, at the same molecular weight, also being apparent in the adrenal gland, DRG and lung tracks of over-exposed auto-

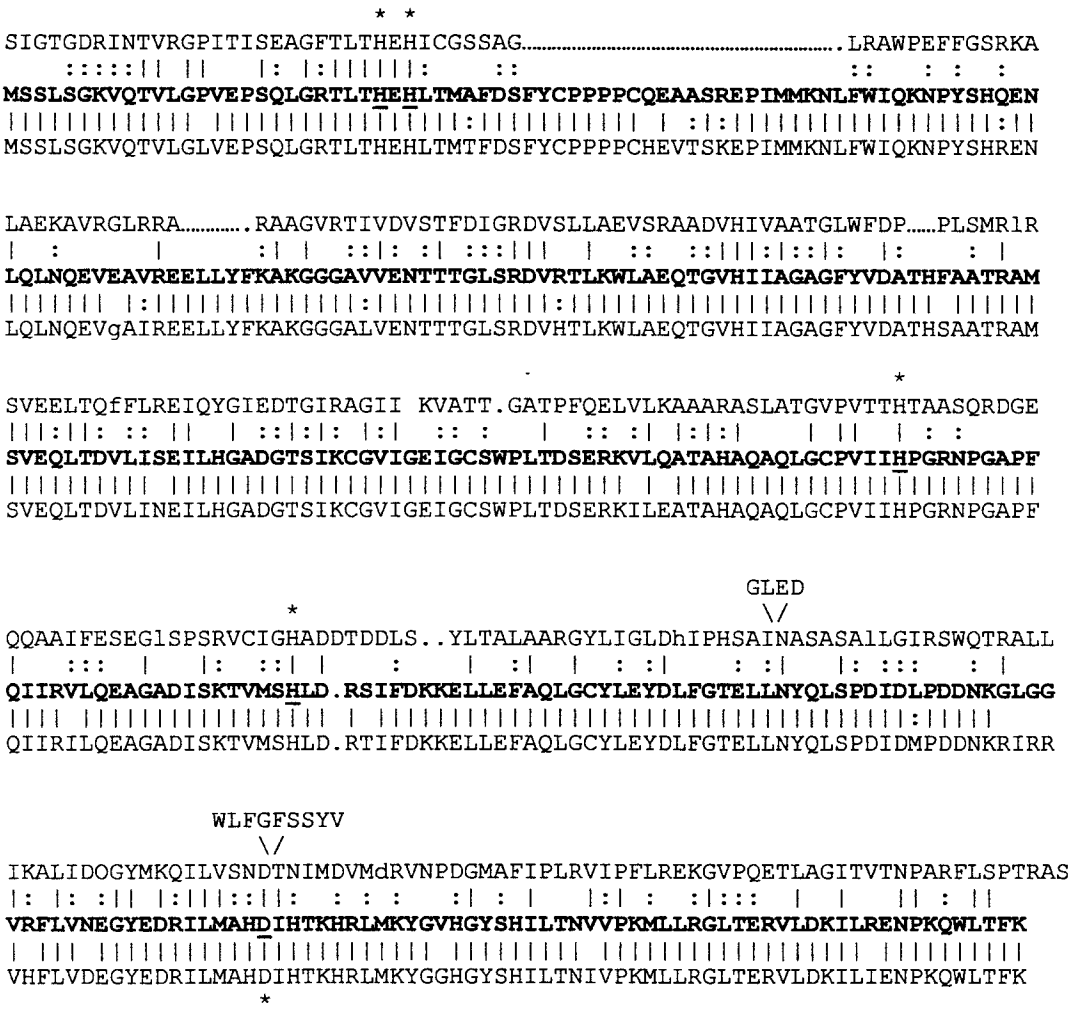

Fig. 2. Comparison of amino acid sequences of *rpr-1* (middle sequence, bold), its murine homologue, *mpr56-1* (bottom) and *Pseudomonas* phosphotriesterase (top). Identity is indicated by '', similarity by ':' (similarity being designated as evolutionary conservative amino acid replacements by the same criteria used by Hou et al. (1995) to characterise *mpr56-1*). The conserved histidine-26, -28, -201, -230, and aspartate-298, essential for function of the bacterial enzyme (see main text), are marked with asterisks.

radiographs. CNS, thymus, liver, heart, muscle and skin were negative.

The distribution of Kid10b/*rpr-1* transcripts within kidney was determined by in situ hybridisation (Fig. 4). Transcripts were detected primarily in the proximal tubule, including Bowman's capsule but not the vascular elements of the glomerulus itself. A fainter signal can also been seen in the other nephric epithelia, but stromal cells do not express the gene at detectable levels. This distribution is similar to that obtained by probing kidney sections with tritiated resiniferatoxin (Fig. 4b); again, nephric epithelia bind the toxin strongly whereas vascular elements of the glomerulus and stroma do so hardly at all.

4. Discussion

4.1. Structure and homologies of rpr-1

We have isolated a novel gene from rat tissues which is so similar to the mouse gene *mpr56-1* that we are confident that they are homologues. We therefore propose that the gene be called *rpr-1*. Despite the fact that *rpr-1* was isolated from a resiniferatoxin-binding clone, there is also no apparent homology between it and the previously described resiniferatoxin-binding protein, RTX-26 [13].

Hou et al. [8] who isolated the murine homologue of *rpr-1*, *mpr56-1*, showed that its closest homology was to a set of microbial genes that includes *Flavobacterium* parathion hydrolase and the phosphotriesterases of *E. coli* and *P. diminuta* [3–5]. These genes code for zinc metallo-enzymes that catalyse

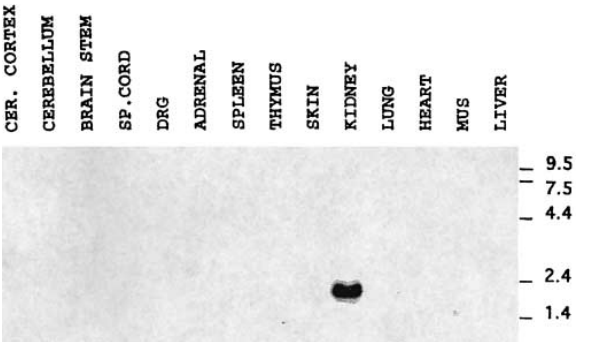

Fig. 3. Northern blot of tissues from new-born rat probed with *rpr-1* cDNA.

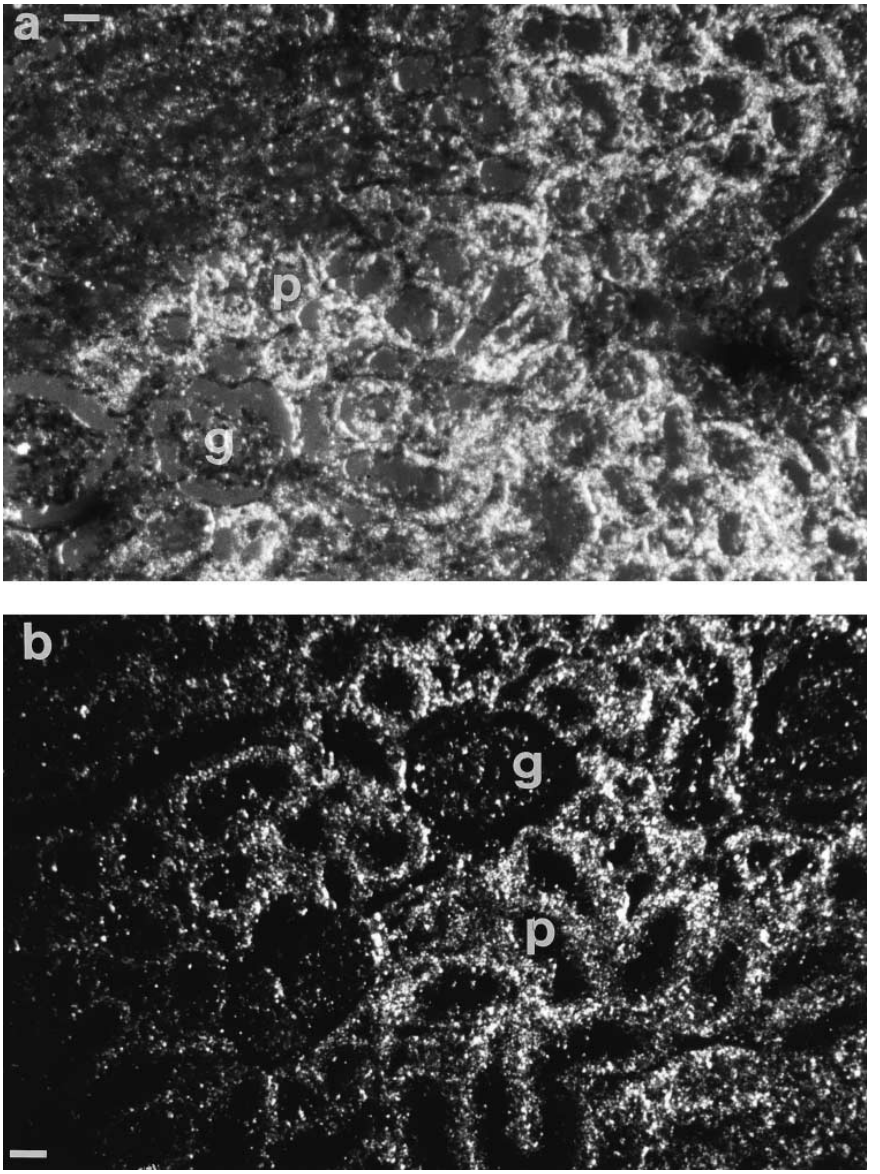

Fig. 4. a: In situ hybridisation of rat kidney sections probed with antisense riboprobes made from *rpr-1* cDNA. A strong signal is present in the proximal tubules (pt), with weaker expression being seen in the other nephric epithelia. The vascular components of the glomerulus (g) show almost no expression. b: Kidney section probed with ^{3}H-RTX; the toxin is bound primarily by tubular epithelia, particularly proximal (p), and only a little by glomeruli (g) and stroma. Scale bar: 20 μm.

the hydrolysis of a range of phosphotriester compounds, including the pesticides parathion and paraoxon and the neurotoxins sarin and soman. The degree of similarity between the rodent sequences and those from bacteria is very high at the active sites of the bacterial enzymes [6,16], strengthening the suggestion that *mpr56-1* and *rpr-1* might also be phosphotriesterases.

The clone carrying *rpr-1* was isolated by screening an expression library with resiniferatoxin. A possible explanation for this might be that the phosphotriesterase homologue can bind to the trialkoxycarbon structure found in resiniferatoxin, which is similar in structure to a phosphotriester but with carbon in place of phosphorus. We have no direct evidence for this, but note that one of the mammalian targets damaged by resiniferatoxin treatment is the kidney [17]. It is therefore possible that the damaging effect of the drug is mediated by binding to *rpr-1* directly in the proximal tubules of the kidney, rather than purely indirectly by the neurally mediated effects that have been proposed to account for this toxicity.

4.2. Expression

The expression pattern of *rpr-1* is intriguing. The only tissue to express *rpr-1* at levels high enough to produce a strong signal on a Northern blot is kidney, specifically proximal tubule epithelium. The renal expression pattern, observed in new-born rats, agrees generally with the expression pattern of *mpr56-1* in new-born mice, reported by Hou et al. [8] but unlike rats, mice also express high level of the transcript in the liver. There are, however, some differences in detail between our rat results and those obtained by Hou et al. in mice; they did not detect expression in lung, and we find very high *rpr-1* expression in neonatal rat kidneys whereas *mpr56-1* appears in murine kidneys only after 1 week post-partum.

4.3. Possible functions of rpr-1

The most outstanding problem to be addressed in future work is that of the in vivo function of *rpr-1*. At present, the only clue comes from its strong homology to bacterial phosphotriesterases, but there is not yet any experimental proof that *rpr-1* (or *mpr56-1*) is itself a phosphotriesterase. Assuming for the moment that it might be, what is likely to be its normal substrate?

Phosphotriesters are not common substrates in biochemistry, but they can be produced by the action of alkylating agents on phosphodiester compounds. These alkylations are generally unwanted and may be deleterious, particularly when they occur in DNA. Alkylating agents such as such as ethyl nitrosurea are known to cause both N-alkylation and O-alkylation, the latter producing a phosphotriester linkage in the normally phosphodiester DNA 'backbone'; in the case of ethyl nitrosurea, more than 50% of the alkylation is of the latter type [18]. Bacteria such as *E. coli* can deal with O-alkylation using the *Ada* protein which, like phosphotriesterases, contains zinc [19,20]. Mammalian cells can also deal with this type of DNA damage, although not particularly efficiently, but the enzymes responsible remain unidentified (see Ref. [21] for a review). Might *rpr-1* might be involved in repair of O-alkylated DNA? If it were, then one might expect that the tissue that expresses it most strongly would be relatively immune from O-alkylating reagents, but unfortunately for this hypothesis rats treated with such agents show a particularly *high* incidence of renal carcinoma [22], the precisely opposite result that would be expected were *rpr-1* to be protective.

Even if it does not act on DNA, a eukaryotic phosphotriesterase might still hydrolyse small cytoplasmic molecules that would otherwise prove toxic; the efficiency of bacterial phosphotriesterases in protecting mice against organophosphate neurotoxins demonstrates the principle well [2]. If this hypothetical function proves correct, eukaryotic phosphotriesterases may turn out to be of great medical and economic significance. Attempts to control the spread of insect-borne diseases, such as malaria, using organophosphate insecticides are being frustrated by the development of resistant strains of insects. Some of these achieve resistance by modification of acetylcholinesterase [23], others show a phosphotriesterase activity apparently different from that of bacterial phosphotriesterases [24], while others achieve resistance by unknown means. If *rpr-1* does turn out to have the phosphotriesterase activity predicted from its homology, it would be very interesting to establish whether insects have homologous genes and whether these genes might confer resistance to pesticides such as *parathion*.

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