These data suggest that PntP2 may be a direct target for Rl/MAP kinase, as has been shown for ETS proteins in vertebrates[9,18]. Indeed, the P2 protein contains a single MAP kinase phosphorylation site (PLTP) motif in the Pointed domain corresponding to the consensus for MAP kinase phosphorylation[19] and can be phosphorylated by Rl/MAP kinase *in vitro* (Fig. 3*a*, lane 2). Phosphorylation is dependent on this single site because a mutant protein ($PntP2^{T151A}$) in which the threonine in the PLTP motif has been replaced by an alanine cannot be phosphorylated *in vitro* (Fig. 3*a*, lane 4). To test whether this mutation affects the function of PntP2 *in vivo*, we generated transgenic lines expressing the mutant transgene under the control of the *sev* enhancer (*sE–pnt*^{T151A}). Not only is this construct unable to rescue the *pnt* phenotype, rather it enhances the mutant phenotype (Fig. 2*g*). Even in a wild-type background the mutant protein prevents neural development of the R7 precursor (Fig. 1*i*) which results in the absence of R7 cells in the adult (Fig. 2*h*). This suggests that the mutant protein competes directly or indirectly with the wild-type protein. Similar to Pnt^{T151A}, a mutant form of vertebrate Elk-1 in which multiple MAP kinase phosphorylation sites have been deleted prevents serum response element-dependent transcription in a dominant-negative fashion[9].

Loss-of-function mutations in *yan*, which encodes another ETS domain protein, result in the recruitment of many R7 cells ever in the absence of *sev* function[11] (Fig. 2*i*). In *yan*, *pnt* double mutants most of the ommatidia lack R7 and some outer photoreceptors (Fig. 2*k*), suggesting that the development of R7 cells in the absence of *yan* function depends on *pntP2* function. Although overexpression of *pntP2* under the control of the *sev* enhancer is not sufficient to transform cone cells into R7 cells in the wild type (Fig. 2*l*), many R7 cells form in a heterozygous *yan* background (Fig. 2*m*). These observations suggest that the mechanisms controlling R7 determination are sensitive to changes in the relative amounts of PntP2 as well as of Yan. The Yan protein contains 10 consensus sites for MAP kinase phosphorylation[19] and, like PntP2, can be phosphorylated by MAP kinase *in vitro* (Fig. 3*a*, lane 6).

Our data thus suggest a model in which MAP kinase activity induces neuronal differentiation by simultaneously inhibiting the Yan repressor and stimulating the PntP2 activator (Fig. 3*b*). The fact that both proteins contain an ETS DNA-binding domain known to recognize conserved target sequences[20] raises the possibility that these two proteins compete directly for binding sites present in the regulatory regions of target genes. □

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Rapid evolution of a protein *in vitro* by DNA shuffling

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DNA SHUFFLING is a method for *in vitro* homologous recombination of pools of selected mutant genes by random fragmentation and polymerase chain reaction (PCR) reassembly[1]. Computer simulations called genetic algorithms[2–4] have demonstrated the importance of iterative homologous recombination for sequence evolution. Oligonucleotide cassette mutagenesis[5–11] and error-prone PCR[12,13] are not combinatorial and thus are limited in searching sequence space[1,14]. We have tested mutagenic DNA shuffling for molecular evolution[14–18] in a β-lactamase model system[9,19]. Three cycles of shuffling and two cycles of backcrossing with wild-type DNA, to eliminate non-essential mutations, were each followed by selection on increasing concentrations of the antibiotic cefotaxime. We report here that selected mutants had a minimum inhibitory concentration of 640 μg ml^{-1}, a 32,000-fold increase and 64-fold greater than any published TEM-1 derived enzyme. Cassette mutagenesis and error-prone PCR resulted in only a 16-fold increase[9].

The poorly hydrolysed antibiotic cefotaxime has a minimum inhibitory concentration (MIC) of only 0.02 μg ml^{-1} for *Escherichia coli* containing the TEM-1 β-lactamase expressed from the vector p182Sfi. The TEM-1 gene was digested into random fragments with DNase I. These small fragments were reassembled into full-length sequences using a PCR-like process and the shuffled sequences reinserted into the vector[1] (Fig. 1). Recombination is caused by the incorporation of a fragment derived from one sequence into another, based on homology. In addition, this method produces a point mutagenesis rate of 0.7%, similar to error-prone PCR[1,13]. This process was repeated for three rounds, and after each round, mutants with improved resistance were selected by plating on increasing levels of cefotaxime. Several hundred colonies from the highest levels of cefotaxime were used as the PCR template for the next round (Fig. 2). Colonies from rounds 1, 2 and 3 were obtained at 0.32–0.64 μg ml^{-1}, 5–10 μg ml^{-1} and 40–80 μg ml^{-1}, respectively. Some colonies from round 3 had a MIC of 320 μg ml^{-1}. Because cefotaxime resistance is cell-density-dependent, the MIC was standardized to 1,000 cells per plate (24 h, 37 °C). At higher cell density, colonies grew at up to 1,280 μg ml^{-1}. A β-lactamase gene (ST-1) of a selected colony contained nine base substitutions, including four silent mutations (Fig. 2).

We attempted to remove all non-essential mutations by backcrossing[1]. ST-1 was shuffled for two rounds in the presence of a 40-fold excess of wild-type DNA fragments (Fig. 2). Small DNA fragments (30–100 bp) were used to increase the efficiency of the backcross. A new β-lactamase gene (ST-2), obtained at 1,280 μg ml^{-1}, had a MIC of 640 μg ml^{-1}. As expected, all four silent mutations had reverted to wild-type sequence. Mutation g4205a, located between the −35 and −10 sites of the β-lactamase P3 promoter, was retained. Sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) analysis of periplasmic extracts showed that ST-1 and ST-2 express 2–3-

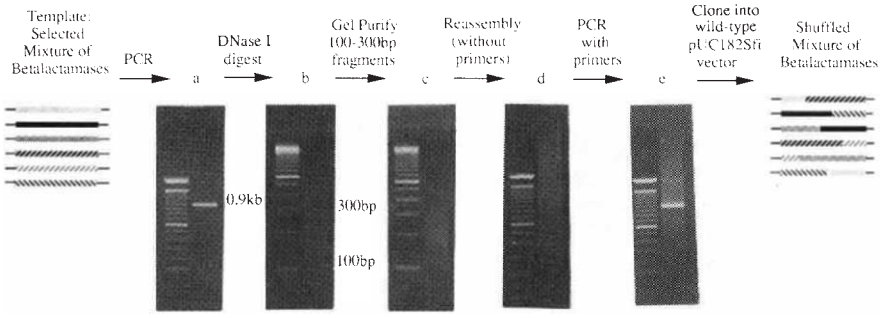

FIG. 1 DNA shuffling of the TEM-1 β-lactamase followed by selection for cefotaxime resistance. A single round of shuffling consists of amplifying the β-lactamase gene by PCR, cutting the PCR product into random fragments with DNase I, gel purification of small fragments, reassembly of the fragments in a PCR-like reaction without primers, amplification of the reassembled product by standard PCR, followed by cloning into the vector and selection on cefotaxime.

METHODS. p182Sfi contains the TEM-1 β-lactamase flanked by *Sfi*I restriction sites. The *Sfi*I sites were added 5′ of the promoter and 3′ of the end of the gene by PCR of the vector with the primers TTCTATTGACGGCCTGTCAGGCCTCAT-ATATACTTTAGATTGATTT and TTGACGCACTGGCCATGGTGGCCAAAAATAAACAAATAGGGGTTCCGCGCACATTT, and by PCR of β-lactamase gene with two other primers listed below. The substrate for the shuffling reaction was dsDNA of 0.9 kb obtained by PCR of p182Sfi with the primers AACTGACCACGGCCTGACAGGCCGGTCTGACAGTTACCAATGCTT and AACCTGTCCTGGCCACCATGGCCTAAATACATTCAAATATGTAT. In rounds 2 and 3 a mixture of >100 cefotaxime^r colonies was used as the template for the PCR. Colony PCR programme: 10 µl of cells in LB broth, 10 min, 99 °C, 35× (94 °C, 30 s; 52 °C, 30 s; 72 °C, 30 s), 5 min, 72 °C. The removal of free primers from the PCR product by Wizard PCR prep (Promega, Madison, WI) was found to be very important. A few µg of the DNA substrate was digested with 0.15 units of DNase I (Sigma) in 100 µl 50 mM Tris–HCl pH 7.4, 1 mM $MgCl_2$, for 10 min at room temperature. Fragments of 100–300 bp were purified from a 2% low melting point agarose gel and resuspended in PCR mix (0.2 mM each dNTP, 2.2 mM $MgCl_2$, 50 mM KCl, 10 mM Tris–HCl pH 9.0, 0.1% Triton X-100) at 10–30 ng µl^{-1}. No primers were added at this point. A PCR programme of 94 °C, 1 min, 40× (94 °C, 30 s; 50–55 °C, 30 s; 72 °C, 30 s), was used in an MJ Research PTC-150 (Watertown, MA) thermocycler. After 40-fold dilution of the minus primer product into PCR mix with 0.8 µM of each primer and 15–20 additional cycles of PCR (94 °C, 30 s; 50 °C, 30 s; 72 °C, 45 s), reproducibly a single product of 900 bp is obtained.

TABLE 1 Characterization of cefotaxime resistance of different combinations of mutations

Name	Genotype	MIC	Source of MIC
TEM-1	Wild-type	0.02	This study
—	E104K	0.08	Ref. 9
—	G238S	0.16	Ref. 9
TEM-15	E104K/G238S*	10	This study
TEM-3	E104K/G238S/Q39K	10*	This study
		2–32	Refs 19, 20
ST-4	E104K/G238S/M182T*	10	This study
ST-1	E104K/G238S/M182T/A18V/t3959a/g3713a/g3934a/a3689g*	320	This study
ST-2	E104K/G238S/M182T/A42G/G92S/R241H/t3842c/a3767g*	640	This study
ST-3	E104K/G238S/M182T/A42G/G92S/R241H*	640	This study

The base numbers (small letters) correspond to the revised pBR322 sequence[24], and the amino-acid numbers (capitals) correspond to the ABL standard numbering scheme[25]. Specific combinations of mutations were introduced into the wild-type p182Sfi by PCR, using two oligonucleotides per mutation. The separate PCR fragments were gel purified and combined by overlap PCR using 10 ng of each fragment. PCR was done for 25 cycles without outside primers, followed by 25 cycles in the presence of the *Sfi*I-containing outside primers. The oligonucleotides for mutation A42G were AGTTGGGTGGACGAGTGGGTTACATCGAACT and AACCCACTCGTCCACCCAACTGATCTTCAGCAT, for Q39K AGTAAAAGATGCTGAAGATAAGTTGGGTGCACGAGTGGGTT and ACTTATCTTCAGCATCTTTTACTT, for G92S AAGAGCAACTCAGTCGCCGCATACACTATTCT and ATGCGGCGACTGAGTTGCTCTTGCCCGGCGTCAAT, for E104K TATTCTCAGAATGACTTGGTTAAGTACTCACCAGTCACAGAA and TTAACCAAGTCATTCTGAGAAT, for M182T AACGACGAGCGTGACACCACGACGCCTGTAGCAATGGCAA and TCGTGGTGTCACGCTCGTCGTT, for G238 alone TTGCTGATAAATCTGGAGCCAGTGAGCGTGGGTCTCGCGGTA and TGGCTCCAGATTTATCAGCAAT, for G238S and R231H (combined) ATGCTCACTGGCTCCAGATTTATCAGCAAT and TCTGGAGCCAGTGAGCATGGGTCTCGCGGTATCATT, for g4205a AACCTGTCCTGGCCACCATGGCCTAAATACAATCAAATATGTATCCGCTTATGAGACAATAACCCTGATA.

*All these mutants additionally contain the g4205a promoter mutation.

fold more β-lactamase than the wild-type plasmid (data not shown). ST-2 contained three of the four amino-acid mutations of ST-1 (E104K, M182T and G238S), as well as three new amino-acid mutations (c3441t resulting in R241H, c3886t resulting in G92S, and g4035c resulting in A42G) and two new silent mutations (t3842c and a3767g).

For comparison with published data, several combinations of mutations were constructed into the wild-type p182Sfi vector (Table 1). All contained the g4205a promoter mutation, and were confirmed by partial sequencing. The known clinical TEM-1-derivatives (TEM-1–19) all contain up to four of a set of eight dispersed mutations[19–21]. Because the maximum MIC obtained by cassette mutagenesis was only 0.64 µg ml^{-1} (ref. 9), high resistance apparently cannot be obtained by mutagenesis of one area. Mutations E104K or G238S are present in published cefotaxime-resistant TEM-1 derivatives (TEM-3, 4, 6, 8, 9, 14–19), and were obtained separately by cassette mutagenesis, with MICs of only 0.08 and 0.16 µg ml^{-1} (ref. 9). In contrast, a combinatorial mutagenesis approach might have yielded the double mutant (TEM-15; ref. 19) with a MIC of 10 µg ml^{-1}. These two mutations thus appear to be synergistic. E104K and G238S in combination with Q39K (TEM-3) or T263M (TEM-4) have reported MICs of 2–32 µg ml^{-1} (refs 19, 21), whereas a TEM-3 like construct in our vector had a MIC of 10 µg ml^{-1}. A construct containing the three amino-acid changes that were conserved after the backcross (E104K, M182T, G238S) also had a MIC of 10 µg ml^{-1}. With or without the silent mutations, constructs containing all of the six amino-acid changes of ST-2 introduced into the wild-type gene yielded colonies with the same MIC as ST-2 (640 µg ml^{-1}). Thus, the six amino-acid mutations (plus the promoter mutation) conferred the high-resistance phenotype.

A control experiment using error-prone PCR but no shuffling resulted in a MIC of only 0.32 µg ml^{-1} after three selection cycles.

A useful approach may be to shuffle many related, naturally occurring genes, such as antibodies[22,23] or homologous genes from different species. The diversity present in such a mixture may be more meaningful than random mutations. □

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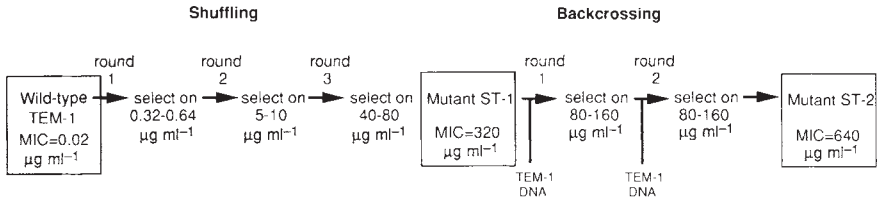

FIG. 2 Three successive rounds of DNA shuffling were done and the cells were grown on increasing cefotaxime levels. The MIC of cefotaxime (Sigma) for *E. coli* XL1-blue (Stratagene, San Diego) carrying wild-type p182Sfi is 0.02 μg ml^{-1}. A mutant with a 16,000-fold increased resistance to cefotaxime was obtained (MIC = 320 μg ml^{-1}). This mutant was backcrossed twice, by shuffling with a 40-fold excess of wild-type DNA. The backcrossed mutant was 32,000-fold more resistant than the wild type (MIC = 640 μg ml^{-1}). After selection, the plasmid of selected clones was transferred back into wild-type XL-1 blue cells to ensure that none of the measured drug resistance was due to chromosomal mutations. DNA sequencing showed that both mutants had 9 single-base-pair mutations.

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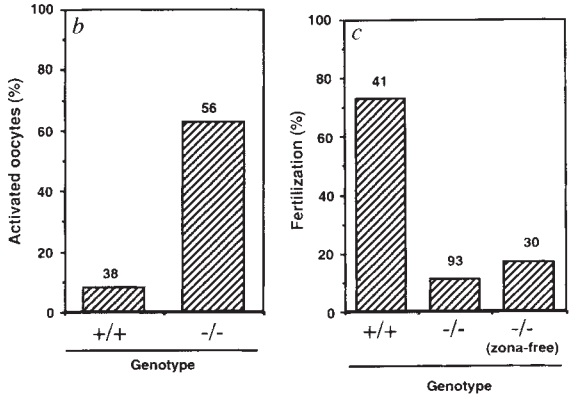
ERRATA

Parthenogenetic activation of oocytes in c-*mos*-deficient mice

Naohiro Hashimoto, Nobumoto Watanabe, Yasuhide Furuta, Hiroyuki Tamemoto, Noriyuiki Sagata, Minesuke Yokoyama, Kenji Okazaki, Mariko Nagayoshi, Naoki Takeda, Yoji Ikawa & Shinichi Aizawa

Nature **370,** 68–71 (1994)

FIGURE 3*b* and *c* of this Letter was an early version that should not have been published. The correct version of this figure is shown here. □



Degradation of trifluoroacetate in oxic and anoxic sediments

Pieter T. Visscher, Charles W. Culbertson & Ronald S. Oremland

Nature **369,** 729–731 (1994)

IN the last sentence of the opening paragraph of this Letter, an error was introduced during editing in which fluoroform was referred to as a "potential ozone-depleting compound." In fact, fluoroform as well as other HFCs were recently shown by Ravishankara *et al.*[1] to have "negligibly small" ozone depletion potentials. □

1. Ravishankara, A. R. *et al. Science* **263,** 71–75 (1994).

Miller-Dieker lissencephaly gene encodes a subunit of brain platelet-activating factor acetylhydrolase

Mitsuharu Hattori, Hideki Adachi, Masafumi Tsujimoto, Hiroyuki Arai & Keizo Inoue

Nature **370,** 216–218 (1994)

THE word 'acetylhydrolase' was accidentally omitted from the end of the title of this paper. The correct title should read "Miller-Dieker lissencephaly gene encodes a subunit of brain platelet-activating factor acetylhydrolase". □