Numerous candidate plasticity-related genes revealed by differential cDNA cloning

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PLASTICITY is a property of the nervous system that allows it to modify its response to an altered input. This capacity for change suggests that there are molecular mechanisms in neurons that can couple stimuli to long-term alterations in phenotype [1–3]. Neuronal excitation elicits rapid transcriptional activation of several immediate–early genes[4], for example c-*fos*, c-*jun* and *zif268*. Many immediate–early genes encode transcription factors that control expression of downstream genes whose products are believed to bring about long-term plastic changes[3,4]. Here we use a highly sensitive differential complementary DNA cloning procedure to identify genes that may participate in long-term plasticity. We cloned 52 cDNAs of genes induced by the glutamate analogue kainate in the hippocampus dentate gyrus. The number of these candidate plasticity-related genes (CPGs) is estimated to be 500–1,000. One of the cloned CPGs (*16C8*), encoding a protease inhibitor, is induced by a stimulus producing long-term potentiation and during dentate gyrus development; a second, *cpg1*, is dependent on activation of the NMDA (*N*-methyl-D-aspartate) receptor for induction and encodes a new small, dentate-gyrus-specific protein. Seventeen of the cloned CPGs encode known proteins, including six suggesting that strong neuronal activation leads to *de novo* synthesis of vesicular and other synaptic components.

As a source of CPG transcripts we chose the hippocampus dentate gyrus (DG), a brain area that participates in plasticity events such as long-term potentiation (LTP)[5], seizures and kindling[6,7], and is involved in learning and memory[8]. To induce CPGs in the DG, rats were injected with kainate, a treatment that induces seizures and LTP-like potentiations[7]. Optimal CPG induction was determined by monitoring kainate effects on proenkephalin messenger RNA levels. Proenkephalin is a suitable marker for CPGs, being encoded by a downstream gene that is controlled by previously induced immediate–early genes, and is induced in the DG by LTP[10] or seizures[11].

CPG cDNAs were operationally defined as complementary to transcripts induced in the DG six hours after kainate treatment. To clone these, we constructed a subtracted cDNA library from the DG of kainate-injected rats, and differentially screened it for kainate-induced cDNAs (Fig. 1*a*). To overcome the limited sensitivity of differential screening, a highly sensitive protocol was used (Fig. 1*b*); allowing detection of ~80% of the library clones.

Duplicate Southern blots containing 25 clones screened as described (Fig. 1*b* legend) are shown in Fig. 1*c*: arrows indicate potential CPG cDNAs, whose inserts produced a stronger signal with the kainate-activated probe (upper blot, +), than with the control probe (lower blot, −). Occasionally clones that appeared to be downregulated by kainate were observed, but these were not investigated further. Induction of positive clones (two independent screens) was confirmed by northern blotting. Examples are shown for three clones marked as kainate-activated (Fig. 1*c*) alongside three other clones and glyceraldehyde 3-phosphate dehydrogenase (G3PD)/proenkephalin controls (Fig. 1*d*). So far, 1,000 clones of the subtracted kainate-activated DG library have been screened: 52 clones (~5%) were of CPGs and were partially sequenced, one third were of known genes (Table 1), and the remainder were new.

LTP is the most popular model for memory-related synaptic plasticity[5]. In most cases of LTP, and in other plasticity paradigms[2,12], glutamate NMDA receptors are thought to mediate induction while non-NMDA receptors participate in maintenance of plasticity[5]. Figure 2*a* shows sample *in situ* hybridizations with two of the new CPGs: *cpg1*, whose induction is blocked by the NMDA-receptor antagonist MK-801, and *cpg2*, whose induction is independent of the NMDA receptor. Induction of 40% of the 52 cloned CPGs depends strictly on NMDA receptor activation (results not shown). CPG 403 (*16C8*; Table 1) was induced in the DG six hours after induction of LTP. Induction levels ranging between those observed for rats 1 and 2 (Fig. 2*b*) were seen only in the DG on the stimulated side (I) and not on the contralateral (C) side. The same CPG (*16C8*) is also expressed during DG development (Fig. 2*c*).

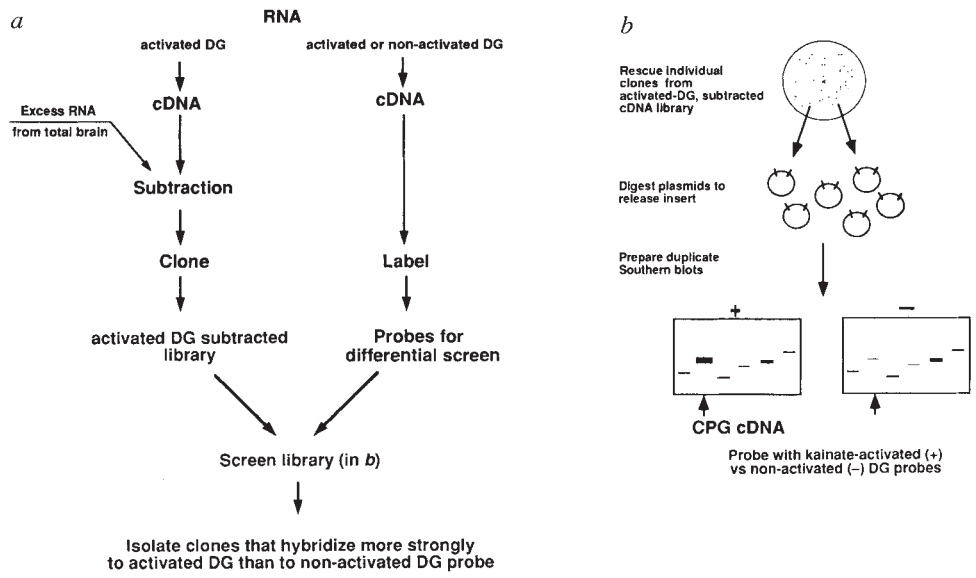
One of the new CPGs, *cpg1*, was highly specific to the adult activated DG (Figs 2*a*, 3*a*), and was dependent on NMDA-receptor activation (Fig. 2*a*) for induction; this CPG was chosen for further analysis. The entire sequence of the major *cpg1* transcript (4,207 base pairs) was determined. The open reading frame (Fig. 3*b*), predicts a small basic protein containing a repeated 6-amino-acid motif (Fig. 3*b*, asterisks). In addition, it contains two cysteine residues at positions 73 and 83, preceded by three amino acids (E, T and K at positions 70–72) that are conserved in the neurotrophin family[13] (Fig. 3*c*).

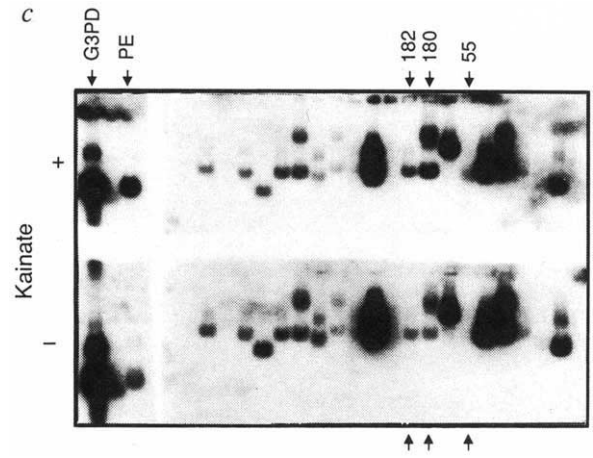
A surprisingly large number of CPGs have been isolated, and the total number of CPGs can be estimated as a result of the high

TABLE 1 Known CPGs and their possible relationship to plasticity

Clone	Product	Function and possible relationship to plasticity	Ref.
Immediate–early genes			
230	c-Jun	Transcription factor	4
284	zif/268	Transcription factor	4
441	c-Fos	Transcription factor	4
514	FosB	Transcription factor	4
639	CREM	Transcription repressor	23
Differentiation and growth response			
263	MyD118	Gene inducible by IL-6 and LIF in myeloid cell differentiation	14, 24
403	16C8	Fibroblast growth factor responsive gene, encodes protease inhibitor TIMP	18, 20
730	ME491	Melanoma associated antigen	25
913	Tyrosine phosphatase	Placenta and brain tyrosine phosphatase	15, 16
Heat-shock proteins			
271	Hsp70	Heat-shock protein stabilizes newly synthesized proteins	17
450	Hsp27	Small heat-shock protein	26
Membrane- , vesicle- and synapse-related			
132	Clathrin heavy chain	Membrane and vesicle traffic	27
180	Secretogranin	Secretory vesicle protein	28
501	Dynorphin	Neuropeptide	29
575	Hsc70	Heat-shock protein homologue involved in uncoating of clathrin from coated pits	30
618	Syndecan	Heparan sulphate proteoglycan core protein	31
777	COMT	Catechol-O-methyl transferase, a catecholamine degrading enzyme	32

All 52 CPG cDNAs were partially sequenced, from both ends and subjected to FASTA searches for sequence homologies. The 17 clones shown shared homologies with the genes indicated. The numbers designating the clones represent their identification numbers during screening. References for the DNA sequences and of possible relationships to plasticity are given in the last column.





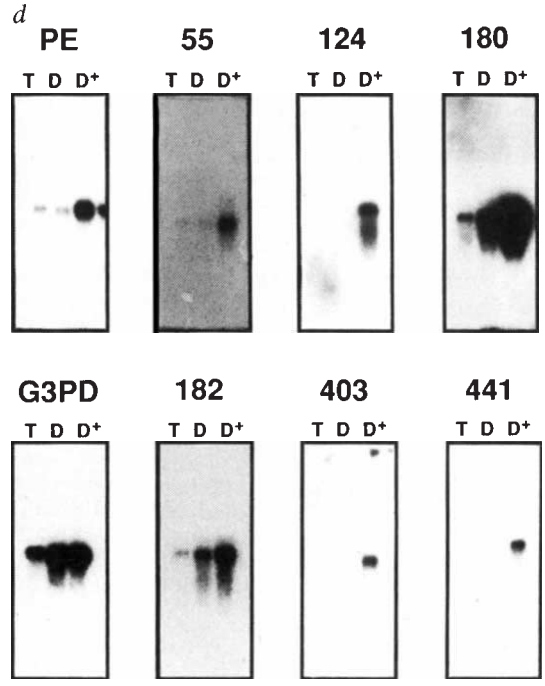


FIG. 1 CPG cDNA cloning approach. *a*. Scheme of subtractive and differential cDNA cloning strategy. Poly(A)$^+$ RNA was extracted from the DG of kainate-treated animals or control animals and used for first-strand cDNA synthesis. Ubiquitous activated DG cDNA sequences were subtracted by hybridization to excess poly(A)$^+$ RNA from total rat brain. The subtracted cDNA was then used to construct a library. First-strand cDNA from activated and non-activated DG was also radioactively labelled to use as probes in the differential screen. Probes were not subtracted to avoid screening artefacts resulting from variability in subtraction efficiency. *b*, Clone-by-clone screening procedure. The two main features of the screen were: (1) individual plaques of the (unamplified) library were picked and 'rescued' as plasmids (pBluescript) from the λZAP vector. Plasmids were restricted to release the cDNA insert and run on duplicate agarose gels; each lane contains DNA of an individual clone. Gels were Southern-blotted and differentially screened for kainate induction. (2) cDNA probes used for the differental screen were labelled to high specific radioactivity and used at very high concentrations. Clones producing a stronger signal with the activated DG probe (left blot) than with the control non-activated probe (right blot), were considered as potential CPGs. *c*, Example of differential screen. Arrows mark three potential CPG clones. On the left are Southern 'strips' used to determine levels of the kainate-inducible proenkephalin (PE) and the housekeeping glyceraldehyde 3-phosphate dehydrogenase (G3PD) cDNAs within the two probes. The PE/G3PD signal ratio reflects the level of kainate activation. Clones giving two signals per lane (for example, 180) arise from incomplete digestion of plasmid DNA. *d*, Northern blots probed with potential CPG clones. Blots were probed with cDNA inserts of potential CPGs, as indicated by arrows in *c*, and with CPGs 124, 403, 441. Poly(A)$^+$ RNA (3 μg) from total rat brain (lanes T), untreated DG (lanes D) and kainate-activated DG (lanes D$^+$) were used, with G3PD and PE acting as controls. All clones positive in two screens were also positive on northern blots; however, 15% of the positives were in low abundance, making visualization difficult.

METHODS. DG RNA: Male rats (Wistar of The Weizmann Institute) of 8–10 weeks old were injected I.P. with kainate (8 mg per kg body weight), killed 6 hours later, and their DG's dissected and stored in liquid N_2. DG's from ~100 animals were pooled, the RNA extracted[33] and selected for poly(A)$^+$, then treated with DNase. Subtracted activated-DG cDNA library: First-strand cDNA was synthesized[34] using oligo(dT)–XbaI primer adapter (Promega). RNA was hydrolysed and the cDNA mixed with total rat brain poly(A)$^+$ RNA and subtracted[35]. Second-strand cDNA was synthesized using terminal deoxynucleotidy transferase to add an oligo(dA) tail to the 3′ side of the first strand, and then oligo(dT)–XbaI primer adapter and Klenow fragment. Double-stranded cDNA was restricted with XbaI, after which it was selected on an agarose gel to include molecules larger >550 bp. The cDNA was ligated into λZAP (Stratagene) arms and packaged (Gigapack, Stratagene). The subtracted library used here has 250,000 PFU (85% recombinants) and the average insert size is 700 bp. Screening: After pBluescript plasmid rescue[36], 2–4 μg plasmid DNA was digested with XbaI and Southern-blotted onto duplicate Hybond N$^+$ filters (Amersham)[37]. The filters were hybridized[37] to cDNA probes (5×10^7–10^8 c.p.m. per ml hybridization solution[37]) for 48 h at 42 °C. First-strand cDNA (100 ng) was labelled using random primers[37] to give a probe of 1.2×10^9 c.p.m. (Cerenkoff).

sensitivity of the screen. Considering that CPGs comprise 5% of the clones screened, and a complexity of ~20,000–30,000 DG RNA species, the total number of CPGs can be extrapolated to 500–1,000. This number may reflect the complex processes induced by activity in DG neurons.

How could the 17 known cloned CPGs (Table 1) contribute to long-term plasticity changes? They can be classified into several categories. The immediate–early genes are induced by stimuli causing plasticity changes[4]. Genes that are induced in other systems during growth or differentiation responses: for example, interleukin-6 and leukaemia inhibitory factor (LIF) induce MyD118 (clone 263) in myelocytes and are also inducers of neuronal differentiation[14]. Tyrosine phosphatases (clone 913) are implicated in development of the nervous system[15,16]. The two heat-shock proteins may relate to increased protein synthesis[17], or to stress effects of kainate. The growth-factor-inducible *16C8* (clone 403; Table 1) encodes a protease inhibitor[18]. This CPG is induced in the DG by LTP-producing stimuli and during plastic phases of DG development[19] (Fig. 2). Tissue-plasminogen activator protease has been reported to be induced by LTP-producing stimuli[20]. The interplay of proteases and their inhibitors is implicated in mechanisms of neurite growth[21], a possible feature of long-term plasticity[2,3,5,7,21].

The most interesting group of known CPGs is that classified as membrane-, vesicle- and synapse-related (Table 1). This sample, although small, indicates that long-term plasticity mechanisms operating under our chosen conditions may involve increased synthesis of presynaptic vesicles (clathrin heavy chain, dynorphin, secretogranin and Hsc70), as well as other synaptic constituents (COMT, syndecan).

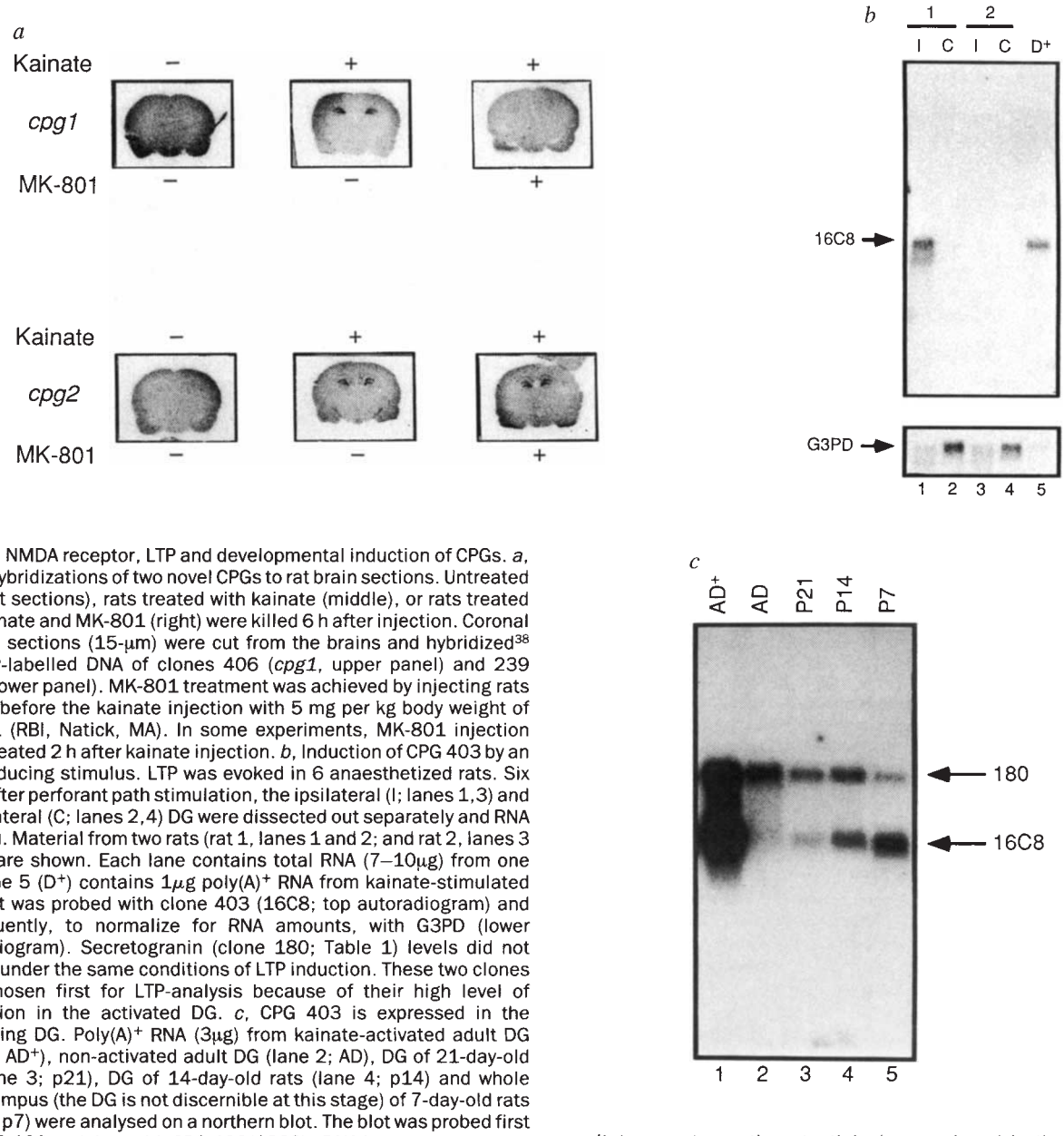
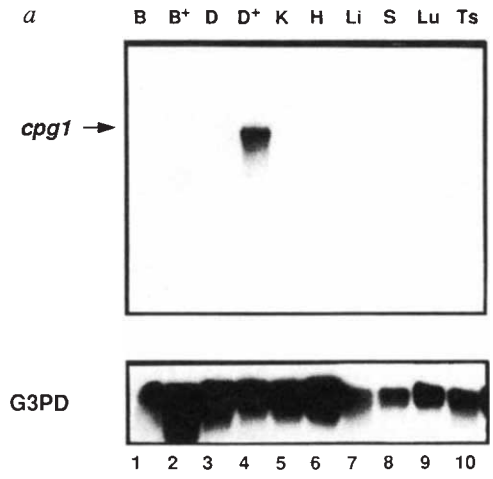


FIG. 2 NMDA receptor, LTP and developmental induction of CPGs. *a*, *In situ* hybridizations of two novel CPGs to rat brain sections. Untreated rats (left sections), rats treated with kainate (middle), or rats treated with kainate and MK-801 (right) were killed 6 h after injection. Coronal cryostat sections (15-μm) were cut from the brains and hybridized[38] with ^{32}P-labelled DNA of clones 406 (*cpg1*, upper panel) and 239 (*cpg2*, lower panel). MK-801 treatment was achieved by injecting rats 30 min before the kainate injection with 5 mg per kg body weight of MK-801 (RBI, Natick, MA). In some experiments, MK-801 injection was repeated 2 h after kainate injection. *b*, Induction of CPG 403 by an LTP-producing stimulus. LTP was evoked in 6 anaesthetized rats. Six hours after perforant path stimulation, the ipsilateral (I; lanes 1,3) and contralateral (C; lanes 2,4) DG were dissected out separately and RNA isolated. Material from two rats (rat 1, lanes 1 and 2; and rat 2, lanes 3 and 4) are shown. Each lane contains total RNA (7–10μg) from one DG. Lane 5 (D^+) contains 1μg poly(A)$^+$ RNA from kainate-stimulated DG. Blot was probed with clone 403 (16C8; top autoradiogram) and subsequently, to normalize for RNA amounts, with G3PD (lower autoradiogram). Secretogranin (clone 180; Table 1) levels did not change under the same conditions of LTP induction. These two clones were chosen first for LTP-analysis because of their high level of expression in the activated DG. *c*, CPG 403 is expressed in the developing DG. Poly(A)$^+$ RNA (3μg) from kainate-activated adult DG (lane 1; AD^+), non-activated adult DG (lane 2; AD), DG of 21-day-old rats (lane 3; p21), DG of 14-day-old rats (lane 4; p14) and whole hippocampus (the DG is not discernible at this stage) of 7-day-old rats (lane 5; p7) were analysed on a northern blot. The blot was probed first with CPG 180 and then with CPG 403(16C8) cDNA inserts.

METHODS. The perforant path was stimulated by a stereotactically positioned electrode in 6 anaesthetized rats. Field potentials evoked by low-frequency stimuli (1–10V, 0.1 ms) were recorded extracellularly before and after inducing LTP. LTP was elicited by 10 consecutive high-frequency trains (0.1-ms pulses of 1,000 μA delivered at 400 HZ for 50 ms). After 30 min, the increases in the slopes of the field excitatory postsynaptic potentials (e.p.s.ps) and in the population spikes were 30–100% and 100–300%, respectively. Rats 1 and 2 gave maximal increases of 95 and 59% in e.p.s.p. slopes, respectively. The variability in CPG 403 induction level among individual animals could stem from variability in the number of fibres tetanized, from varied response levels, or from effects of anaesthesia[5] in different animals.



b

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ATGGCCAAGAAGTCACATCCTTACCTACACTGGTATAAGAACGAAAACGGCCCATCAGTA
M  A  K  K  S  H  P  Y  L  H  W  Y  K  N  E  N  G  P  S  V      20
                                                  ********
ATCAGGCCTGGAGGGAGGAGAGGTTCAGGGAGCCCTAGCTCTACCGCTGCTCTCTTTGTC
I  R  P  G  G  R  R  G  S  G  S  P  S  S  T  A  A  L  F  V      40
*******
TCTGTAGATGCAAGCAGAGGGCGAACATTACCTTCAGTTATACACCCCTTGGTGACCAAA
S  V  D  A  S  R  G  R  T  L  P  S  V  I  H  P  L  V  T  K      60
                            ****************
TCTAAAGGTCACACAGATGGTCCTGGTGAAACGAAATGCTCATTAAACCAAACCCCAAGC
S  K  G  H  T  D  G  P  G  E  T  K  C  S  L  N  Q  T  P  S      80

CACGTTTGTGGAAAGGAACTGTTAGGGACAAGGGGAGGGGGA
H  V  C  G  K  E  L  L  G  T  R  G  G  G      94
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c

NGF:	ETKCRAPNPVESGC
NT3:	ETRCKEARPVKNGC
BDNF:	ETKCNPMGYTKEGC
CPG1:	ETKCSLNQTPSHVC

FIG. 3 Characterization of the novel CPG *cpg 1*. *a*, Tissue distribution. Northern blot containing 5 μg poly(A)$^+$ RNA from total rat brain of untreated (B), kainate-treated animals (B$^+$), untreated DG (D), kainate-activated DG (D$^+$), kidney (K), heart (H), liver (Li), spleen (S), lung (Lu), and testis (Ts) was probed with clone 406 (*cpg1*) cDNA and subsequently with a G3PD probe (lower panel). *b*, Predicted amino-acid sequence of the *cpg1* product. Over 30 independent cDNA clones were mapped and used to determine the sequence of the major *cpg1* transcript and a few minor variants. There were at least 4 modes of alternative splicing in the 5′ untranslated region, none of which affected the predicted open reading frame. The sequence of the open reading frame and the predicted protein sequence are shown in single-letter amino-acid code. Two pairs of basic amino acids are underlined. A repeated amino-acid motif (PSVI[R/H]P) is indicated by asterisks. *c*, Cysteine residues conserved between the neurotrophin family and the predicted *cpg1* product. Sequences of the nerve growth factor (NGF), neurotrophin (NT3) and brain-derived neurotrophic factor (BDNF)[13] are compared to the predicted *cpg1* product (CPG1) at residue positions 70–83. Cysteines are underlined and the conserved preceding 3 amino acids are shown in bold.

METHODS. To obtain clones spanning the full-length *cpg1* transcript, two additional (non-subtracted) cDNA libraries were constructed from activated DG RNA as described for Fig. 1. One library was constructed using internal primers derived from sequences at the 5′ side of the original clone 406 and was selected to include cDNAs larger than 2 kb. The second library was constructed with an oligo(dT)–*Not*I primer adapter (Promega) in order to span artificial termination of clones resulting from internal *Xba*I site(s). Screening the activated and size-selected DG libraries with clone 406 resulted in 179 positives. Clones spanning the entire sequence were isolated and sequenced on both strands using Sequenase (USB).

Kainate causes not only seizures and potentiations but also glutamate excitotoxity[7,22]. The DG, however, is the hippocampal region least prone to damage by kainate[22]. Furthermore, 15 of the 17 known CPGs cloned (Table 1), appear to be relevant to plasticity. First, the immediate–early genes are induced by plasticity-producting stimuli[4]; second, the differentiation and proliferation response genes are not associated in other systems with cell death; finally, the vesicle and synapse building blocks do not implicate neuronal toxicity but rather synapse remodelling.

Our study describes highly sensitive differential cDNA cloning of a large number of CPG cDNAs, and predicts the existance of ten times as many more. The CPGs include known genes (for example, those encoding 16C8, secretogranin, clathrin heavy chain) and new genes (such as *cpg1*) not previously considered as potential participants in long-term plasticity. The extent of overlap between the different mechanisms underlying various CNS plasticity phenomena is not known[2,3], but one way to clarify this issue would be to define the panel of CPGs induced in each case. □

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Characterization and localization of the *FMR-1* gene product associated with fragile X syndrome

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THE fragile X syndrome is the most frequent form of inherited mental retardation after Down's syndrome, having an incidence of one in 1,250 males[1,2]. The fragile X syndrome results from amplification of the CGG repeat found in the *FMR-1* gene [3–6]. This CGG repeat shows length variation in normal individuals and is increased significantly in both carriers and patients[3–6]; it is located 250 base pairs distal to a CpG island[6] which is hypermethylated in fragile X patients[4–7]. The methylation probably results in downregulation of *FMR-1* gene expression[8]. No information can be deduced about the function of the *FMR-1* protein from its predicted sequence. Here we investigate the nature and function of the protein encoded by the *FMR-1* gene using polyclonal antibodies raised against the predicted amino-acid sequences. Four different protein products, possibly resulting from alternative splicing, have been identified by immunoblotting in lymphoblastoid cell lines of healthy individuals. All these proteins were missing in cell lines from patients not expressing *FMR-1* messenger RNA. The intracellular localization of the *FMR-1* gene products was investigated by transient expression in COS-1 cells and found to be cytoplasmic. Localization was also predominantly cytoplasmic in the epithelium of the oesophagus, but in some cells was obviously nuclear.

As a first step in the identification and characterization of the *FMR-1* gene product, antibodies were raised against different regions of the predicted amino-acid sequence of the *FMR-1* protein[3]. Two different methods were used. A complementary DNA fragment of *FMR-1* containing nucleotides 940–1,325 was cloned in the *Escherichia coli* expression vector pGEX[9] and antibodies were raised in rabbits against the *FMR-1* fusion protein (α765). The second approach was to use a synthetic oligopeptide corresponding to the carboxy-terminal end (position 632 to 656) of the *FMR-1* protein[3] as antigen (α1079)[10].

These antibodies were then used to analyse the *FMR-1* protein (FMRP) in lymphoblastoid cell lines from patients (n=5) and controls (n=3). FMRP was immunoprecipitated with α765 and analysed by immunoblotting. Four species (M_rs 74K, 72K, 70K and 67K) (Fig. 1*A*) that were present in the controls were absent in four of five patients. The lack of cross-reactive

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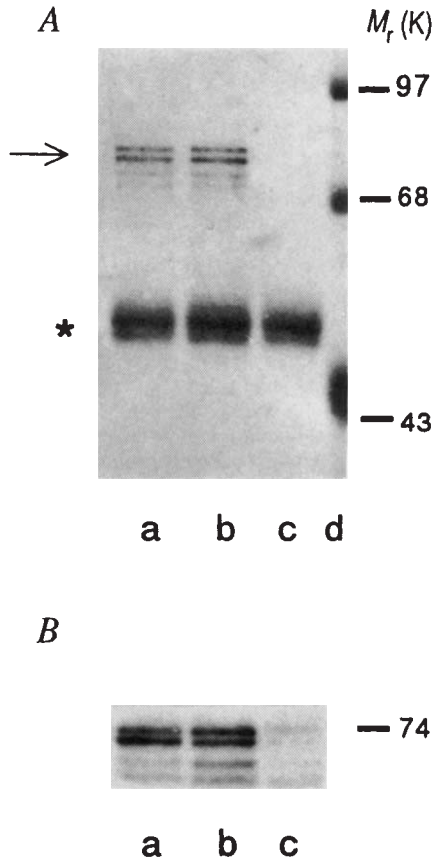

FIG. 1 Immunoprecipitation of lymphoblastoid cell lines with α765 antibodies. *A*, Control cells: lanes a (C49) and b (ROS31) and fragile X patient cells in lane c (RJK1411). Sizes of the protein bands are 74, 72, 70 and 67K. Lane d, molecular weight standards. Nonspecific band (IgG) is indicated by an asterisk, the position of FMRPs is indicated by an arrow. *B*, Control cells: lane a (ROS31) and b (GM7381), cells from a mosaic patient lane c (RJK2368). Molecular weight of upper band indicated on the left.
METHODS. Polyclonal antibodies were raised in rabbits against a GST (glutathione *S*-transferase)–*FMR-1* fusion protein obtained using the pGEX plasmid expression system[9]. A cDNA fragment containing nucleotides 940 to 1,325 of the coding and 3,557–3,765 of the non-coding sequence of the previously published *FMR-1* cDNA[3] was cloned in the *Eco*RI site of pGEX-3X. Expression of the construct in the protease-deficient *E. coli* strain B121 resulted in the synthesis of a *FMR-1* polypetide of 134 amino acids fused with the C terminus of Sj26, a 26K glutathione *S*-transferase. The *FMR-1* polypetide consisted of amino acids 314 to 442 of the *FMR-1* gene plus five additional amino acids (Cys-Thr-His-His-Leu) as a result of the cloning procedure. Expression and purification of the fusion protein were as described[9]. Antibodies were affinity-purified using columns of GST and (GST)-*FMR-1* fusion protein successively. Pellets of lymphoblastoid cell lines were homogenized in a buffer containing 10 mM HEPES, 300 mM KCl, 100 μM $CaCl_2$, 5mM $MgCl_2$, 0.05% Tween and 0.45% Triton X100, pH 7.4. Cell homogenates were spun down for 10 min (10,000*g*). The supernatants were incubated with protein A–Sepharose (Pharmacia) for 2 h at 4°C to remove the IgG present in the lymphoblastoid cells. Protein A–Sepharose was spun down and the supernatant was incubated overnight with α765 and protein A–Sepharose at 4° C. After washing (3×) the immunoprecipitate, sample buffer was added and the precipitates were electrophoresed in SDS–polyacrylamide (8% polyacrylamide gel, 1% crosslinking) and electroblotted. Polypeptides were visualized using 1,000× diluted α765 and a 1,000× diluted alkaline phosphatase-conjugated goat anti-rabbit IgG as a second antibody. Polypeptides were detected using naphthol AS-MX phosphate and 4-aminodiphenylamine diazonium sulphate or AMPPD (TROPIX) as substrates.