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Differential Amplification of Intron-containing Transcripts Reveals Long Term Potentiation-associated Up-regulation of Specific Pde10A Phosphodiesterase Splice Variants*

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We employed differential display of expressed mRNAs (Liang, P., and Pardee, A. B. (1992) *Science* 257, 967–971) to identify genes up-regulated after long term potentiation (LTP) induction in the hippocampus of awake adult rats. *In situ* hybridization confirmed the differential expression of five independently amplified clones representing two distinct transcripts, cl13/19/90 and cl95/96. Neither cl13/19/90 nor cl95/96 showed significant sequence homology to known transcripts (mRNA or expressed sequence tag) or to the mouse or human genome. However, comparison with the rat genome revealed that they are localized to a predicted intron of the phosphodiesterase Pde10A gene. cl13/19/90 and cl95/96 are likely to be part of the Pde10A primary transcript as, using reverse transcriptase-PCR, we could specifically amplify distinct introns of the Pde10A primary transcript, and *in situ* hybridization demonstrated that a subset of Pde10A splice variants are also up-regulated after LTP induction. These results indicate that amplification of a primary transcript can faithfully report gene activity and that differential display can be used to identify differential expression of RNA species other than mRNA. In transiently transfected Cos7 cells, Pde10A3 reduces the atrial natriuretic peptide-induced elevation in cGMP levels without affecting basal cGMP levels. This cellular function of LTP-associated Pde10A transcripts argues for a role of the cGMP/cGMP-dependent kinase pathway in long term synaptic plasticity.

Memory, the recollection of acquired information, is encoded in the brain as enduring changes in distributed neural networks. Synapses that support activity-dependent changes in synaptic efficacy are therefore a likely location of information storage in the brain. Long term potentiation (LTP)[1] is an activity-dependent long lasting increase in synaptic efficacy in the hippocampus and other cortical structures and is the main cellular model for learning and memory (2). There is strong evidence indicating that LTP constitutes a neural substrate for some forms of learning and memory (3): (i) both LTP and learning depend on the activation of similar signal transduction cascades (4); (ii) animal models in which the induction of LTP is impaired are generally also impaired in hippocampal-dependent learning and memory (see, *e.g.*, Refs. 5–8, but see Ref. 9); (iii) mice that show enhanced LTP also show enhanced learning and memory (10, 11); and (iv) behavioral paradigms can induce an increase in the synaptic strength in the amygdala (12).

Another point of congruence is the requirement for *de novo* RNA and protein synthesis both for the formation of long term memory (13, 14) and the late stage of LTP, L-LTP (15–17). The observation that L-LTP requires *de novo* RNA and protein synthesis has led to a search for genes that are modulated by LTP. To date, several transcripts have been identified and include both transcription factors (*e.g. Zif268*) (18–20), as well as proteins with a cellular and/or synaptic function (20–26). In addition to *de novo* RNA and protein synthesis, LTP may also be associated with the induction of novel splice isoforms (27, 28). Although not all regions of the hippocampal formation have a similar transcriptional response to electrical stimulation (29, 30), experiments in which expression or function of LTP-associated transcripts was perturbed has demonstrated their importance for the expression of (L-)LTP (8, 31–35). Moreover, expression of LTP-modulated transcripts such as *Zif268* and *Arc/Arg3.1* appears to be both associated with specific learning tasks (36–39) and required for the formation of long term memories (8, 33–35). These proteins and their associated biological functions therefore are beginning to provide an insight into the molecular bases of the long term changes underlying synaptic plasticity and memory consolidation.

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) AY462091–AY462095

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[1] The abbreviations used are: LTP, long term potentiation; L-LTP, late stage of long term potentiation; RT, reverse transcription; ECS, electroconvulsive shock; ANP, atrial natriuretic peptide; eYFP, enhanced yellow fluorescent protein; cGK, cGMP/cGMP-dependent kinase; EPSP, excitatory postsynaptic potential.

In this study we employed differential display of expressed mRNAs (1) to identify genes induced by LTP in the awake animal. Although differential display is primarily aimed to identify changes in expression levels of mRNA, our analysis highlighted the ability of this technique to identify differential expression of other RNA species. Using *in situ* hybridization, we were able to confirm the differential expression of five clones that represent two independent transcripts, cl13/19/90 and cl95/96. We provide evidence demonstrating these clones are part of the primary transcript (*i.e.* RNA prior to excision of introns) of Pde10A, a gene encoding a cGMP phosphodiesterase (40–43). Our results argue for a role of the cGMP/cGMP-dependent kinase (cGK) pathway in long term synaptic plasticity.

EXPERIMENTAL PROCEDURES

In Vivo Recordings—Eighty-five male Sprague-Dawley (Iffa Credo, France) rats weighing between 300 and 350 g were prepared for chronic unilateral recording in the dentate gyrus as described previously (44). Detailed experimental protocols used in this study for the induction and recording of LTP in the awake rat have been described (25). All experiments were conducted in accordance with the recommendations of the EU directive (86/609/EEC) and the French National Committee (87/848).

Differential Display—Differential display experiments were performed as described (25). The primers that led to the differential amplification of *cl13/19/90* and *cl95/96* were: Cl19, ACGACTCACTATAGGGC$(T)_{12}$CG and ACAATTTCACACAGGACGACTCCAAG; Cl13, ACGACTCACTATAGGGC$(T)_{12}$CG and ACAATTTCACACAGGACGACTCCAAG; Cl90, ACGACTCACTATAGGGC$(T)_{12}$AG and ACAATTTCACACAGGACGACTCCAAG; Cl95, ACGACTCACTATAGGGC$(T)_{12}$CA and ACAATTTCACACAGGACTTTCTACCC; and Cl96, ACGACTCACTATAGGGC$(T)_{12}$CA and ACAATTTCACACAGGACTTTCTACCC.

Electroconvulsive Shock (ECS)—ECS was induced in 20 rats following light halothane anesthesia. Ear-clip electrodes were used to deliver 200 V (sine wave) at 50 mA for 2 s to induce clonic seizure according to published protocols (74). Animals were subsequently allowed to recover before being sacrificed 1, 3, 6, 12, or 24 h after ECS ($n = 4$ per group). Control rats ($n = 6$) were subjected to equivalent handling without subsequent stimulation. All brains were removed, frozen on dry ice, and stored at −80 °C prior to subsequent analysis by *in situ* hybridization.

In Situ Hybridization—Coronal sections (14 μm) were mounted on polylysine-coated Superfrost slides and stored at −80 °C. *In situ* hybridization was performed essentially as described elsewhere (29). Briefly, sections were thawed, fixed in 4% paraformaldehyde, acetylated in 1.4% triethanolamine and 0.25% acetic anhydride, dehydrated through graded ethanol solutions, and delipidated in chloroform. Sections were hybridized overnight at 42 °C in 100 μl of buffer containing 50% formamide, 4× SSC, 10% dextran sulfate, 5× Denhardt's solution, 200 mg/ml acid alkali-cleaved salmon testis DNA, 100 mg/ml long chain polyadenylic acid, 25 mM sodium phosphate (pH 7.0), 1 mM sodium pyrophosphate, and 100,000 cpm radiolabeled probe (~1 ng/ml) under parafilm coverslips. Sections were washed in 1× SSC at 55 °C (30 min), 0.1× SSC at room temperature (5 min), and dehydrated in ethanol. Sections were then exposed to autoradiographic film. [^{35}S]ATP-end-labeled probes (PerkinElmer Life Sciences) were generated using terminal deoxynucleotidyltransferase (Promega) according to instructions from the manufacturer. A 50-fold excess of unlabeled oligonucleotide was used as a negative control; for probes cl13/19/90, cl95/96, cl77, and WS, labeled sense oligonucleotides served as an additional control.

Antisense sequences were: Cl13/19/90, AGCCATTTCCCAGATGCTCGCCCAGTATCTTTGAAAGGGGTCAG; Cl95/96, ATGGCAGAAGGCAGGGAAAGCAGGTTGGCAGGAGAAGAAGGCTGA; Cl77, AGCACGCACAAAGCATTCTGGGATTTCTGCCTCTGCGGGTCTTGA; WS, TCTCTGGCTCAAGACTTGGCAAGACAGGTGGTTCAGGAGAGGTGA; YB, GAAGCAACTCGCATTGTTAGAGGGTCCATCTTCCATGTCGGAG; YC, TCAGGAATGTTCAGGACTTCTCCCGTTCTTGCCACTTGACCAG; YD, GGCTTCCAGTCCATGTGCAGTGGTCCTCTGTAGGCATCCATG; YE, AAACCAGGCAGTGGGTTCCTCCTCTGGGAGCTGGGGTTCTC; and YF, CCGCCAAAGTATCCTCCAGTACAAAACTCCTTTCTGCTTCATT.

Labeled sections were exposed to high resolution film (Kodak BiomaxMR). Autoradiographs were analyzed by measuring the integrated density relative to a ^{14}C standard using NIH Image or SAMBA. The signal intensity in stimulated dentate was expressed as a percentage of the unstimulated contralateral dentate granule cell layer. ECS activates gene expression on both sides of the brain; in these experiments, *Pde10A* expression was compared with the levels in sham-handled, and age-matched controls. The statistical significance of any differences following LTP or ECS treatment as compared with respective controls was assessed using Student's *t* test.

RT-PCR—Total RNA was isolated from adult rat hippocampus, cerebellum, cortex, and olfactory bulb using TRIzol (Invitrogen) according to instructions from the manufacturer. 10 μg of total RNA was treated with 4 units of DNase for 15 min. at 37 °C. 3 μg of DNase-treated RNA samples were then reverse transcribed for 1 h at 50 °C in the presence of 200 units of Superscript III (Invitrogen), 150 ng of oligo$(dT)_{18}$ primer, 0.5 mM dNTPs, 50 mM DTT, and RNase inhibitor in a total volume of 20 μl. A control reaction was performed in absence of reverse transcriptase; control samples were otherwise treated identically.

0.2 μl from the reverse transcribed cDNA was used for each RT-PCR reaction. Primer pairs used for detection of the primary transcript were (from 5′ to 3′ end of the Pde10A gene): GTGGTTGTGTGACATTTTCCATCC and GACCTTGGGTTTGTGCTGTTTC (primers o-p), ATCCCGACCCTTGGCTTCTCAATG and TGCTTGTCTGCTTTCTTCCCTG, TTGCTCCCTGGTGCTGTTTG and TCCTCCATTGCTCTCAGAAGCC, GGTTCGTTTGCTGTTGGCTTAG and TATTACACCCATCCCGCTCCTC (primers m-n), GGATAGACCTAAGGCAACCACAAG and TCACAATGGCTTCTGACACGC, AGGATGCAGTCCATGATGGTGG and ACTTAAACCCTTGGTCTCTGCTAGG (within cl95/96), TGAAGCCCCAGCACTACACAACTC and CCATTCTTTCTCCCTCCAGACATAC (primers k-l), TTCTCAGCCATCTTCTCCCACC and TCTGCCTTTTTTCTCCCTTTGG, GGGGATAATAGCTCAAGGGAGACC and AGCACAGAATCCAGAAGAGGGTATC (primers i-j), TATGCGAAAGCACCAGTCGG and TTCTCTTCACACAGTAGCACCCTG, TGGAATGGCATCGGCTTGTAGC and TGGTCCTCATAATCCTGTGTCTGC (primers g-h), ACTGAGGCTGGAGGAGTTAGAGTTC and TAGAAAGATGGGGCAGGTGGTC, CAAAGAAGTTGTCTGGGGTGTGC and TTCATCCTCAGTCAAGGGCATCGG. Primers used to estimate relative levels of LTP-associated splice isoforms: TTTCCCAGAGACTTGTCCCTACG and GGCTTTTGTGGCTATCCTGATG (primers b-c), AACTCCGCCCCTATGTCAAGTG and TACACGACTCCCTGCATGTTCG (primers a-d). Primers used to amplify full-length Pde10A3 were TTAAAGGTGGGAGCAAAGGCCC and ATCAGTCATCGACCTTCCTGGTCG, those used to amplify full-length Pde10A11 were ATGTCGAAGAAGCGCAAAGCCCTC and ATCAGTCATCGACCTTCCTGGTCG.

cAMP and cGMP Measurements—Sequence-verified full-length rat Pde10A3 and Pde10A11 were cloned into pcDNA3.1 (Invitrogen). Cos7 cells, grown on 6-well plates, were transfected with the Pde10A constructs or peYFP-C1 (Clontech) using DEAE dextran (45). Prior to cAMP and cGMP measurement, transfection efficiency was monitored as the percentage of cells that expressed eYFP monitored with a fluorescent microscope. In our hands, this method routinely transfects ~70–80% of cells in culture. 18–24 h following transfection, cAMP and cGMP measurements were performed (cAMP or cGMP enzyme immunoassay biotrack system, Amersham Biosciences) according to instructions from the manufacturer. Levels were expressed relative to the total amount of protein in each sample. Protein concentrations were measured using a BCA protein assay kit (Pierce) according to the instructions from the manufacturer. cGMP experiments are averages of five values obtained in two independent experiments, cAMP experiments are averages of four values (four independent experiments) for each construct.

RESULTS

Identification of LTP-associated Transcripts—Tetanic stimulation of the perforant path in freely moving rats induced a stable form of LTP, measured by the mean increase in the slope of evoked EPSPs, that persisted at least 6 h without decrement (Fig. 1*A*). Control rats receiving a similar number of stimuli at low frequency did not display such increase in EPSP slope (*CS* group; data not shown). Animals were sacrificed 1, 3, and 6 h after tetanic stimulation, or 1 h after control stimulation, and RNA was extracted from the dorsal half of the dentate gyrus ipsilateral to the implanted electrodes. RNA extracted from the dorsal half of the dentate gyrus from a group of naive animals served as an additional control (*naive* group). We next performed differential mRNA display (1) using the Hieroglyph mRNA profile kit (Genomyx) to screen for LTP-modulated transcripts (Fig. 1*B*). Candidate LTP-modulated transcripts were then subjected to a second screening round on independently generated brains using *in situ* hybridization. *In situ* hybridiza-

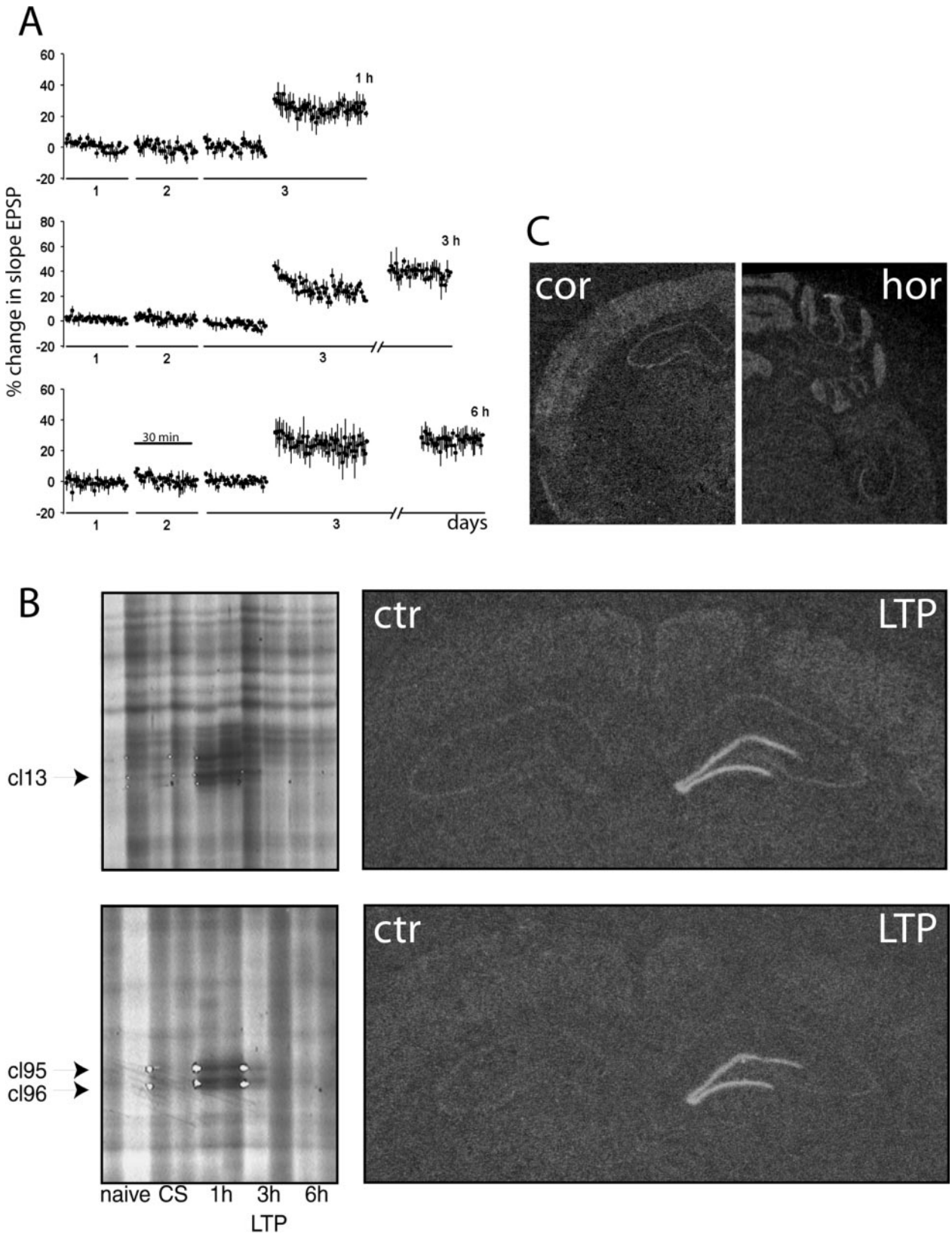

FIG. 1. **Differential display of gene expression in LTP.** *A*, tetanization of the perforant pathway in freely moving adult rats results in a stable form of LTP that persisted at least 6 h without decrement. Control rats not receiving high frequency stimulation did not display such increase in slope EPSP (data not shown). *B*, RNA extracted from the dorsal half of the stimulated dentate gyrus was used to screen for LTP-regulated transcripts. Differential display analysis yielded many candidate differentially expressed clones (see labeled examples). *In situ* hybridization confirmed the LTP-associated expression of five clones that represent two independent transcripts (cl13/19/90 and cl95/96). These transcripts are up-regulated 1 h after the induction of LTP. *C*, highest basal expression levels of these clones is found in the cerebellum as determined by *in situ* hybridization on horizontal (*hor*) sections derived from adult rat brain (examples obtained with probes directed against cl13/19/90; cl95/96 has an identical spatial distribution; data not shown). Other brain regions with detectable expression levels of cl13/19/90 and cl95/96 include the dentate gyrus, areas CA1 and CA3 of the hippocampal formation, and olfactory bulb. *cor*, coronal section of adult rat brain; *ctr*, control.

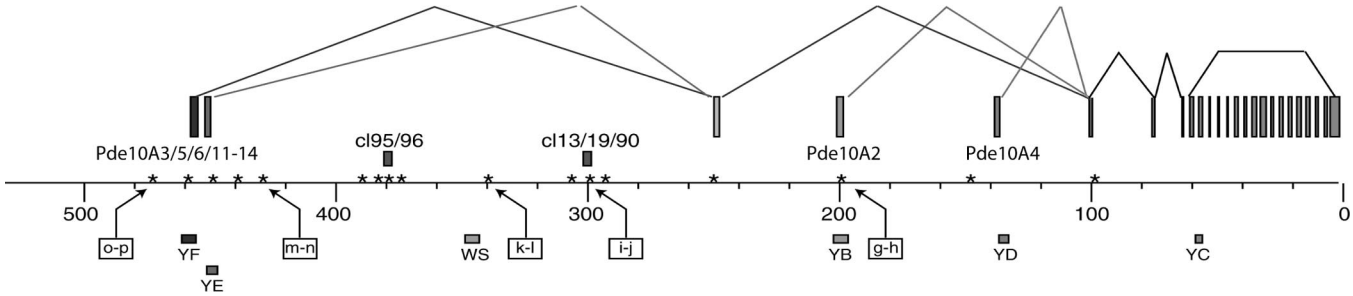

FIG. 2. **Genomic organization of the rat Pde10A gene.** Depicted are exons (not to scale) of various Pde10A splice variants on a ~500-kb region of rat chromosome 1. *, sites at which RT-PCR experiments were performed. All RT-PCR reactions were designed to amplify intronic sequence. *g-h*, *i-j*, *k-l*, *m-n*, and *o-p* are RT-PCR primer pairs as used in Fig. 3. *YC*, *YD*, *YE*, *YF*, and *WS* are sites probed in the *in situ* hybridization experiments displayed in Figs. 4 and 5. *Scale bar* indicates position of exons relative to the last exon of Pde10A (kb).

tion was performed using radiolabeled 45-mer oligonucleotides complementary to the sequence of differentially regulated transcripts. From this secondary screen we confirmed differential expression of five clones: cl13, cl19, cl90, cl95, and cl96. Sequence identity between these clones indicated that they represent two independent transcripts (Fig. 1*B*): cl13/19/90 and cl95/96. Both clones were strongly up-regulated 1 h after the induction of LTP and returned to pre-tetanus levels 3 h following tetanization ($n = 4$ for each time point). No up-regulation was observed in the dentate gyrus of animals receiving a pseudo-tetanus or those in which the tetanus was delivered in the presence of the NMDA receptor antagonist 3((*R*)-carboxypiperazin-4-yl)-propyl-1-phosphonic acid (10 mg/kg, $n = 4$ for each condition, data not shown).

The two transcripts exhibit a similar spatial distribution as determined by *in situ* hybridization. In the adult rat, under basal, non-tetanized conditions, cl95/96 and cl13/19/90 are expressed at highest levels in the cerebellum. Other regions with detectable expression levels include the olfactory bulb, the dentate gyrus, areas CA1–CA3 of the hippocampal formation, and cortex (Fig. 1*C*). In addition to their similar spatial distribution, both cl13/19/90 and cl95/96 are expressed at similar low levels. Expression levels can be estimated by *in situ* hybridization as the time required to develop a signal on autoradiographic film. Approximately 3 months of exposure was required for cl13/19/90 and cl95/96 to reach quantifiable signal intensities on autoradiographic film compared with ~4 days of exposure for *Zif268* (see also Ref. 29), indicating ~20–30-fold lower expression levels.

cl13/19/90 and cl95/96 Both Lie within an Intron of the Pde10A Gene—As our differential display screen identified only a partial cDNA sequence, we set out to determine the nature of the complete transcript of cl13/19/90 and cl95/96. Cl13/19/90 and cl95/96 appear to lie in a non-coding part of the full-length transcript as they do not contain any large open reading frames. Interestingly, Northern blot analysis failed to identify a full-length cl13/19/90 or cl95/96 transcript (results not shown) whereas other mRNAs using the same blots were readily detectable (see, *e.g.*, Ref. 46). The size of the full-length mRNA of cl13/19/90 or cl95/96 therefore remained elusive. We next compared the cDNA sequence of cl13/19/90 and cl95/96 to the public domain sequence data bases. To our surprise we did not detect significant homology to (i) the non-redundant data base that contains mRNA sequence, (ii) the data base of expressed sequence tags, or (iii) the data bases of the completed human or mouse genome. The lack of homology of cl13/19/90 and cl95/96 to these data bases therefore did not help determine the nature of their complete mRNA transcript.

The cDNA sequence of cl13/19/90 and cl95/96 did, however, show a clear homology when compared with the draft genome sequence of the rat, the species used for our differential display screen. Interestingly, both cl13/19/90 and cl95/96 lie in close proximity (80 kb) to each other on chromosome 1. Neither clone contain introns themselves as they match contiguously on the genome. Although no confirmed transcripts are present in close (<40 kb) vicinity, both clones are situated within the same large (200 kb) intron of the Pde10A gene (Fig. 2). The absence of any information regarding the identity of the full-length transcript of cl13/19/90 and cl95/96 therefore led us to hypothesize these clones may be part of the gene in which they are localized. In theory, cl13/19/90 and cl95/96 could be part of the Pde10A gene either as part of its primary transcript or as a novel splice variant. However, it is also possible cl13/19/90 and cl95/96 are not part of the Pde10A gene at all. Our next experiments were performed to discriminate between these options.

We first hypothesized that, if cl13/19/90 and cl95/96 are part of the Pde10A primary transcript, we should be able to detect other regions of the primary transcript. We therefore performed RT-PCR on intronic regions of the Pde10A gene indicated by an *asterisk* in Fig. 2 using DNase-treated, reverse transcribed RNA derived from adult rat hippocampus and cerebellum. Interestingly, all RT-PCR reactions confirmed the presence of intronic Pde10A sequence (examples are shown in Fig. 3), with the notable exception of a reaction performed outside the Pde10A gene (primers o-p, Figs. 2 and 3). The amplified fragments include introns that lie 3′ to the common intron that encompasses cl13/19/90 and cl95/96. The PCR products were not the result of amplification of genomic DNA, as they were only observed using reverse transcribed RNA and not in control reactions using non-reverse transcribed RNA. Identical results were obtained using RT-PCR on cDNA derived from adult rat cerebellum (results not shown). Because amplification of other intronic regions of the Pde10A gene may not have been detectable if cl13/19/90 and cl95/96 encode either a novel Pde10A splice variant or are part of a separate gene, our results favor the hypothesis that cl13/19/90 and cl95/96 are part of the Pde10A primary transcript.

We next hypothesized that, if cl13/19/90 and cl95/96 are part of the Pde10A gene, the gene itself should show an identical expression pattern and LTP-dependent modulation. We therefore performed *in situ* hybridization on the Pde10A gene using sections of rat brains in which LTP was induced and followed for 1 h. Because the Pde10A gene is subjected to alternative splicing (41, 42, 47, 48), we performed *in situ* hybridization on several of these variants (oligonucleotide probes YC–YG, location indicated in Fig. 2). Because exon Ib (see Fig. 6) is part of both Pde10A3 and Pde10A6 splice variants, *in situ* probe YF cannot distinguish between these isoforms. Similarly, exon II is part of both Pde10A5 and Pde10A11 splice variants and therefore *in situ* probe YE cannot distinguish between these two isoforms.

Indeed, *in situ* hybridization confirmed the LTP-associated up-regulation of several Pde10A splice isoforms (Fig. 4); Pde10A3/A6 and A5/A11 are both up-regulated following LTP

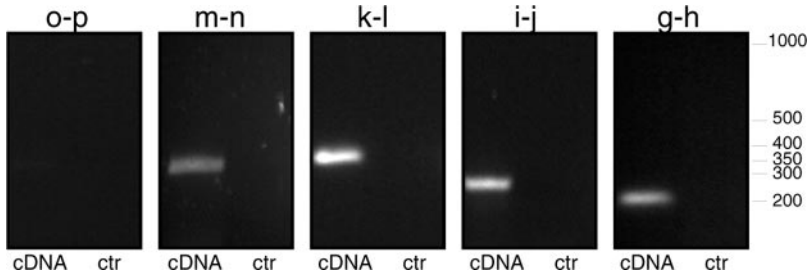

FIG. 3. **RT-PCR performed on various intronic regions of the Pde10A gene amplified fragments of predicted length.** We were unable to amplify fragments in a region outside the Pde10A gene (*o-p*). These results suggest the primary transcript of Pde10A can be detected by RT-PCR. PCR products were only observed using reverse transcribed RNA (*cDNA*); no product was detected when using non-reverse transcribed RNA (*ctr*). This indicates the PCR products were not the result of amplification of genomic DNA. Identical results were obtained using RT-PCR on cDNA derived from adult rat cerebellum (data not shown).

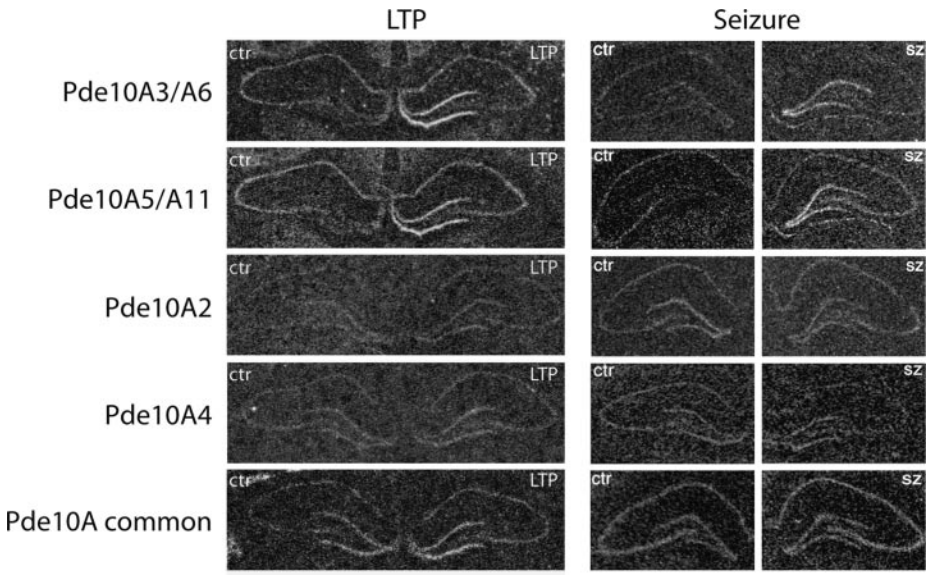

FIG. 4. **LTP-associated up-regulation of specific Pde10A splice variants.** *In situ* hybridization directed against individual Pde10A splice variants demonstrates Pde10A3/A6 and Pde10A5/A11 are up-regulated following the induction of LTP ($n = 4$) and maximal seizures ($n = 3$, probes YE and YF, respectively; Fig. 2); splice variants Pde10A2 and Pde10A4 are not (probes YG and YD). A region common to all splice variants (probe YC) shows a modest LTP-associated up-regulation that can be expected if it reflects expression of both LTP-associated and non-activity-dependent splice variants. *ctr*, control; *sz*, seizure.

induction (probes YE and YF, respectively, indicated in Fig. 2) whereas Pde10A2 and A4 are not (probes YG and YD). The LTP-associated expression was observed in the dentate gyrus ipsilateral to the side of stimulation ($n = 4$). No up-regulation was found in the contralateral hippocampus ($n = 4$) or in animals in which the electrodes were implanted without electrical stimulation ($n = 2$, data not shown). A detailed analysis of all LTP-associated Pde10A splice variants is given in Fig. 6. A region common to all splice variants (probe YC) shows a modest LTP-associated up-regulation, which can be expected if it reflects expression of both LTP-associated and non-activity-dependent splice variants. To confirm these observations, we performed similar experiments on tissue obtained 1 h after the induction of a maximal seizure (see "Experimental Procedures"). Seizures have been reported to cause a stronger transcriptional response than LTP (20) and have been used to identify genes modulated by electrical activity (20, 21, 23, 49). Indeed, *in situ* hybridization confirmed that not only cl13/19/90 and cl95/96 (results not shown), but also Pde10A3/6 and Pde10A5/11 show seizure-associated up-regulation (Fig. 4, $n = 3$). Pde10A2 and Pde10A4 are not up-regulated following seizure; if anything, there was a trend toward down-regulation.

Based on the short half-life of primary transcripts, the expression levels of a gene can be expected to be higher than that of its primary transcript. Indeed, individual Pde10A splice variants had signal intensities ~20-fold stronger than those for cl13/19/90 and cl95/96, determined by the time required to reach comparable signal intensities on autoradiographic film. Our observations that (i) specific Pde10A splice variants are up-regulated following LTP induction, (ii) these splice variants show an identical spatial expression pattern compared with cl13/19/90 and cl95/96, and (iii) expression levels of Pde10A splice variants are markedly higher than those of cl13/19/90 and cl95/96 favor the hypothesis that cl13/19/90 and cl95/96 are part of the Pde10A primary transcript.

If cl13/19/90 and cl95/96 are indeed part of the Pde10A primary transcript, we hypothesized that an *in situ* hybridization experiment directed at a random intronic sequence of the Pde10A gene should give not only identical activity-associated up-regulation but also similar levels of expression as cl13/19/90 and cl95/96. *In situ* hybridization performed against a random intronic Pde10A sequence (probe WS, the location of which is depicted in Fig. 2) indeed showed both seizure-associated up-regulation and a very low level of expression (Fig. 5, $n = 3$). Similar to cl13/19/90 and cl95/96, probe WS required ~1-month exposure time on autoradiographic film before expression was detected. Our findings that probe WS (directed against an arbitrary selected intronic sequence) and cl13/19/90 and cl95/96 have similar expression levels argues against the possibility that cl13/19/90 and cl95/96 encode novel splice variants.

Characterization of LTP-associated Pde10A Splice Variants—Putative amino acid sequences have been described for some of the LTP-associated Pde10A splice variants (Pde10A3, Pde10A5, and Pde10A6) (41). The cDNA sequences of the splice variants used for the design of oligonucleotide probes were obtained by means of data base comparison to the reverse translated predicted protein sequences. In the public domain data bases, several transcripts were present that exhibit sequence identity to the Pde10A splice variants, Pde10A3, Pde10A5, and Pde10A6 (41). These splice variants all share a

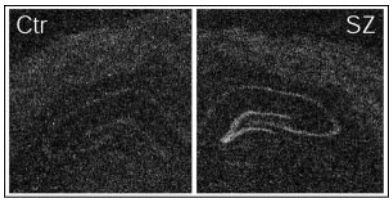

FIG. 5. ***In situ* hybridization performed against a random intronic Pde10A sequence (probe WS, see location in Fig. 2) showing activity-associated up-regulation.** As with cl13/19/90 and cl95/96, probe WS required ~1 month of exposure time on autoradiographic film before expression was detected. Because the expression levels of a primary transcript is likely to be lower than that of its mRNA, this finding favors the possibility that cl13/19/90 and cl95/96 are part of the Pde10A primary transcript and do not encode novel splice variants. *Ctr*, control; *SZ*, seizure.

common 5′ region, which indicates they may be derived from a single promotor. To estimate the relative levels of individual splice variants, we performed RT-PCR on RNA isolated from adult rat hippocampus, cerebellum, cortex, and olfactory bulb (all regions expressing cl13/19/90 and cl95/96). RT-PCR using primers a–d yielded two distinct bands of approximately 520 and 390 bp, the expected fragment size for Pde10A3 and Pde10A6, respectively (Fig. 6). Of the two, Pde10A3 (GenBank™ accession no. AY462095) appears to be most abundant. RT-PCR using primers b-c also yielded two distinct bands of approx. 840 and 720 bp (Fig. 6). Again, the largest and most abundant fragment corresponded to the expected size for Pde10A3. Sequence analysis identified the lower fragment as a novel splice variant, which we coined Pde10A11 (GenBank™ accession no. AY462091). Additional sequence analysis on all products generated by the RT-PCR reaction revealed several additional novel Pde10A splice variants, Pde10A12–A14 (accession nos. AY462092, AY462093, and AY462094). These fragments, although detectable by RT-PCR, are clearly not as abundant as Pde10A3, -A6, or -A11. In Fig. 6 we present a model of splicing of the first four exons of the LTP-associated splice variants of the Pde10A gene. The relative levels of individual splice variants were identical in the different brain regions examined.

The large number of splice variants give rise to several protein products of which Pde10A3, Pde10A5, Pde10A11, and Pde10A12 have unique amino acid sequences. Pde10A6, Pde10A13, and Pde10A14 do not possess such unique N-terminal amino acid sequence, as they have translational start sites downstream of exon IV (in a region common to all Pde10A isoforms). A weak splice donor sequence was found in exon Ib; splicing may occur at various sites at the 3′ end of this exon (sequence TTGTACTGGAGêGATACTTTGGCGGêCAGê, with splice sites indicated with ê). The putative protein sequence of splice variants using this exon is not affected because it is part of the 5′ UTR.

The splice variants modulated by LTP all had exons I and/or II as upstream exons, suggesting that these transcripts shared a common promotor. Our observations that the relative levels of Pde10A3, Pde10A6, and Pde10A11 are similar between the various brain regions examined (as determined by RT-PCR) and that the relative levels of Pde10A3/A6 and Pde10A5/A11 splice variants remain similar following LTP induction (as determined by *in situ* hybridization, Fig. 4) argues against the possibility of regulated splicing between the LTP-associated splice variants.

Pde10A3 Expression Reduces Elevations in cGMP Concentrations—Because Pde10A is likely to function as a cyclic nucleotide phosphodiesterase, we examined the effect of Pde10A expression on cellular cAMP and cGMP levels. Full-length rat Pde10A3 (the most abundant LTP-associated Pde10A splice isoform, see Fig. 6) was transiently transfected into Cos7 cells. cGMP content was measured using a cGMP enzyme immunoassay, and expressed relative to the total amount of protein in each well. As can be seen in Fig. 7, expression of Pde10A3 did not affect basal levels of cGMP as compared with control (eYFP)-transfected cells. When Cos7 cells were stimulated for 10 min with 10^{-7} M atrial natriuretic peptide (ANP), a stimulus that induces cGMP production (50, 51) control (eYFP)-transfected cells responded with a ~2-fold increase in cGMP levels (28.1 ± 2.4 and 57.7 ± 5.2 fg of cGMP/g of protein, $p < 0.001$). In contrast, Pde10A3-transfected Cos7 cells did not respond to ANP with an increase in cGMP levels (26.9 ± 5.5 and 32.2 ± 9.6 fg of cGMP/g of protein, $p < 0.05$ ANP-stimulated Cos7 cells eYFP *versus* Pde10A3). These results suggest that Pde10A3 functions as a cGMP phosphodiesterase but only on elevated cGMP levels. Because Pde10A can hydrolyze both cAMP and cGMP (40, 42, 43), we also measured effects of Pde10A expression on cAMP levels. Full-length rat Pde10A3 or Pde10A11 was transiently transfected into Cos7 cells. As can be seen in Fig. 7, expression of the LTP-associated Pde10A isoforms Pde10A3 or Pde10A11 affected neither basal nor forskolin-stimulated cAMP levels compared with control (eYFP)-transfected cells. No difference was observed using either Pde10A3 or Pde10A11 ($n = 4$ for each); data are therefore combined in Fig. 7. These results suggest that, although Pde10A can hydrolyze cAMP *in vitro*, in intact cells it does not affect cAMP levels. Alternatively, the forskolin-stimulated cAMP levels may not be sufficiently high to reach the K_m for cAMP of Pde10A3.

DISCUSSION

We have employed differential display of expressed mRNAs (1) to identify genes induced by LTP in the hippocampus of awake rats. This study identified ~150 candidate-regulated genes that were subjected to a second screening round using *in situ* hybridization. We confirmed the differential expression of five LTP-regulated clones representing two independent transcripts, cl13/19/90 and cl95/96.

Neither cl13/19/90 nor cl95/96 showed significant sequence homology to any known transcript (mRNA or expressed sequence tag) in mouse, rat, or human data bases, or to any genomic sequence in mice or humans. The two clones did, however, show sequence identity to two genomic sequences in the rat, both localized to the same intron of the Pde10A gene. Our evidence suggests that cl13/19/90 and cl95/96 are part of the Pde10A primary transcript rather than parts of a distinct uncharacterized gene within the intron because: (i) randomly selected primers can detect all introns examined of the Pde10A primary transcript using RT-PCR and *in situ* hybridization; (ii) Pde10A splice variants (Pde10A5/A11 and Pde10A3/A6) are also up-regulated following the induction of LTP (the probes we used did not allow us to distinguish between A5 and All, and between A3 and A6; however, we can infer that at least two of the splice variants A3, A5, A6, and A11 were up-regulated); (iii) cl13/19/90 and cl95/96 have an identical expression pattern to the LTP-associated splice variants of Pde10A; (iv) expression levels of cl13/19/90 and cl95/96 are markedly lower than that of Pde10A mRNA as expected from the relatively short half-life of the primary transcript, and (v), other regions of the primary transcript show identical LTP-associated up-regulation and expression levels. Furthermore, cl13/19/90 and cl95/96 show other features that can be expected if they are part of a primary transcript: (i) Northern blot analysis using cl13/19/90 or cl95/96 failed to identify any full-length transcript, (ii) cl13/19/90 and cl95/96 do not contain a large open reading frame, and (iii) cl13/19/90 and cl95/96 are themselves intronless.

Our experiments highlight the potential use of differential display to identify changes in expression levels of RNA species

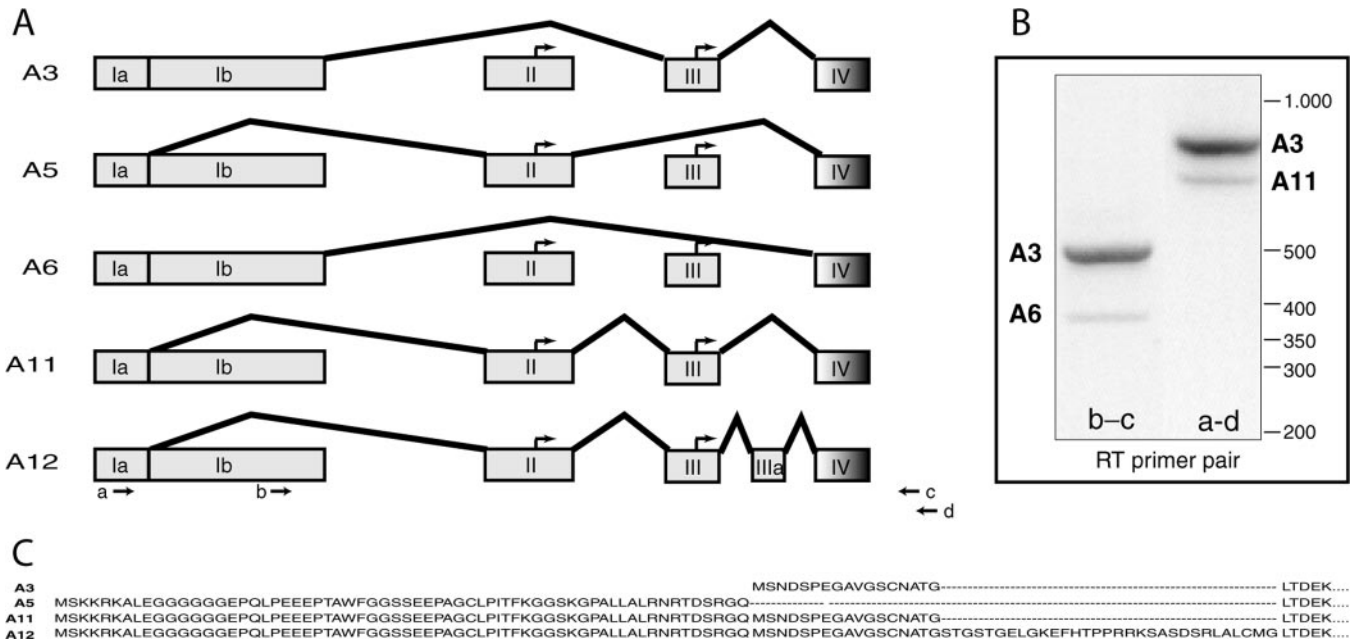

FIG. 6. **Relative abundance of LTP-associated splice variants.** *A*, splicing of the first four exons of the LTP-associated splice variants of the Pde10A gene (*top left*). All LTP-associated splice variants share a common 5′ exon, suggesting they may be derived from a common promotor. Not depicted are Pde10A13 and Pde10A14; they are derived by combining exons Ia-Ib-IV-V- (skipping exons II and III) and Ia-Ib-VIII-IX- (skipping exons II-VII), respectively. *Arrows* in exons II and III indicate translational start sites. *B*, RT-PCR was performed to estimate the relative levels of individual splice variants. RT-PCR primers a–d (position indicated in *A*) yielded two distinct bands of approximately 520 and 390 bp, the expected fragment size for Pde10A3 and Pde10A6, respectively. Of the two, Pde10A3 appears to be most abundant. RT-PCR using primers b and c also yielded two distinct bands of approximately 840 and 720 bp. Again, the largest and most abundant fragment corresponded to the expected size for Pde10A3. Sequence analysis identified the lower fragment as a novel splice variant, we coined Pde10A11. The relative levels of individual splice variants were identical in the different brain regions examined. *C*, the predicted unique amino acid sequence of LTP-regulated splice variants. LTDEK represents start of the protein sequence present in all (regulated and non-regulated) Pde10A isoforms. The translation initiation site for Pde10A5, -A11, and -A12 is located in exon II; for Pde10A3 it is located in exon III.

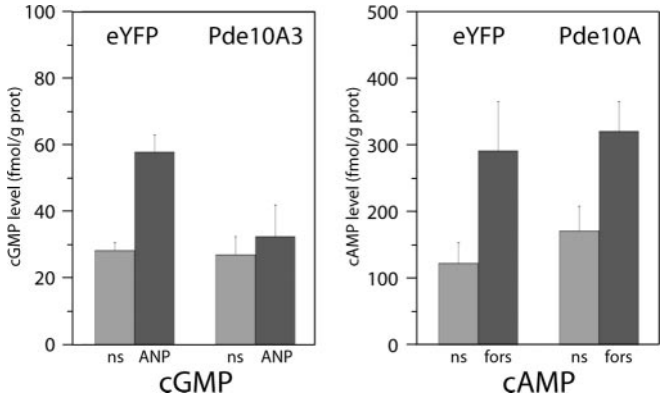

FIG. 7. **Regulation of cyclic nucleotides by Pde10A3.** Left: Transient transfection of Pde10A3 into Cos7 cells reduces the ANP-induced cGMP elevation. ANP (10^{-7} M) was applied for 10 min. Basal cGMP levels (*ns*) were similar in Pde10A3-transfected and eYFP-transfected cells. However, Pde10A3-transfected Cos7 cells did not respond to ANP with an increase in cGMP levels, whereas control cells responded with a ~2-fold increase in cGMP levels ($p < 0.001$, stimulated cells compared with non-stimulated cells; $p < 0.05$, stimulated control cells compared with stimulated, Pde10A3-transfected cells). *Right*, transient expression of the LTP-associated Pde10A isoforms Pde10A3 or Pde10A11 neither affected basal nor forskolin-stimulated cAMP levels compared with eYFP control-transfected cells. Forskolin (*fors*, 10^{-5} M) was applied for 30 min. Forskolin induced an increase in cellular cAMP levels both in eYFP- and Pde10A3/A11-transfected cells ($p < 0.05$ for both, compared with corresponding non-stimulated cells). No difference was observed using either Pde10A3 or Pde10A11 ($n = 4$ for each), and data have therefore been combined.

other than mRNA. Why our differential display screen identified Pde10A primary transcript in preference to its mRNA remains to be determined. It is possible that the nature of the experiment (*i.e.* number of amplification cycles) favored the detection of low abundant transcripts. Alternatively, large introns have a relatively long half-life because introns have to be transcribed in full before they can be spliced out. The half-life of an intron is therefore determined by its length and by the rate of RNA polymerase II transcription. The latter has been estimated to range from ~1–2.5 kb/min (28, 55), suggesting that the 200-kb Pde10A intron in which cl13/19/90 and cl95/96 are situated requires at least 80 min to be transcribed in full. Significantly, our results indicate that levels of the primary transcript can be used to study the activity of a gene.

Although we provide evidence that cl13/19/90 and cl95/96 are part of the Pde10A primary transcript, we cannot exclude the possibility that they are part of a large intron retained in the mRNA. Intron retention can form a mechanism to generate alternative splice variants and results in the presence of intronic sequence within mRNA (see, *e.g.*, Refs. 52–54). It should be noted that all examined introns can readily be detected by RT-PCR (see Figs. 2 and 3), an observation that argues against retention of a specific intron. However, because intron retention may be a regulated process (53), it remains possible that cl13/19/90 and cl95/96 are part of an intron that is retained in an activity-dependent manner. Such a transcript would form a minority of mRNA species, as the expression levels of cl13/19/90 and cl95/96 are markedly lower than those of predicted exons. It should also be noted that, for Pde10A3, the translational start site lies downstream of the putatively retained intron and may not affect the Pde10A protein product.

Because our screen identified clones upstream from the poly(A^+) tail, it follows that the primers used to generate cDNA hybridized to an alternative sequence. There are several poly(A^+) stretches within the Pde10A primary transcript that can form priming sites for reverse transcription. This priming site forms the 3′ end of our differentially expressed clones, and was identified as agctcCGAAAAAAAAAAAAaagaaaaa (5′ to 3′) for cl13, cl19, and cl90, and gaccaaTGAAAAAAAAggAggccg for

cl95 and cl96 (caps denote nucleotides complementary to the RT-primer). These sequences are complementary to the last 14 (cl13 and cl19) and 10 nucleotides (cl95 and cl96) of the RT primer. The priming site for cl90 has one mismatch with the genomic sequence at the penultimate base. These short genomic poly(A^+) stretches, when part of the primary transcript, are therefore apparently sufficient to serve as priming sites for reverse transcription.

The Pde10A gene is a member of the phosphodiesterase gene family, which contains over 11 subfamilies (PDE1–11), encoded by at least 19 different genes (56, 57). Of these, PDE4 has also been shown to be associated with electrical stimulation (26, 58). Pde10A can hydrolyze both cAMP and cGMP (40, 42, 43), although the enzyme is suggested to have a higher specific activity for cGMP (40). Our data confirm the hypothesis that Pde10A functions as a cGMP phosphodiesterase; our data also show that in Cos7 cells Pde10A3 only affects stimulated increases in cGMP concentrations, whereas it does not reduce basal cGMP levels. These findings are likely to be related to the relatively low affinity of Pde10A for cGMP. We also found that neither Pde10A3 nor Pde10A11 affects cellular cAMP levels. Although our data suggest Pde10A does not function as a cAMP phosphodiesterase, it remains possible that the forskolin-stimulated cAMP levels achieved in these experiments were not sufficiently high to reach the K_m for cAMP. It has been postulated that cAMP inhibits cGMP hydrolysis (43). Pde10A may therefore function as a cAMP-inhibited cGMP phosphodiesterase (40, 43), and it is possible therefore that cGMP signaling will be affected in cells with an increased expression of Pde10A.

One postulated function of cGMP is to act in presynaptic terminals as a downstream effector of the retrograde messenger NO via activation of a soluble guanylate cyclase (59). Downstream effectors of cGMP include cGMP-dependent protein kinase I and II, both of which are expressed in the hippocampus (60, 61). Several studies have provided evidence for a role of the NO-cGMP pathway in synaptic plasticity: (i) inhibitors of nitric-oxide synthase, guanylyl cyclase or cGKs can block the induction of LTP (62–67); (ii) exogenous NO paired with a brief tetanus can generate a stable NMDA receptor-independent potentiation (Refs. 66 and 68, but see Ref. 69); (iii) a hippocampal specific deletion of cGK-I diminished LTP in adult mice (70); and (iv) brief perfusion of 8-br-cGMP before weak tetanic stimulation produces long lasting potentiation (65, 66). Together these data suggest that stimulation of the NO-cGMP pathway facilitates synaptic plasticity, whereas inhibition of this pathway depresses it. Our finding that the cGMP phosphodiesterase Pde10A gene is up-regulated following the induction of LTP is likely to act as a damper on further episodes of LTP. Interestingly, it was recently reported that LTP is associated with a prolonged reduction in cGMP levels (71), and it is tempting to speculate that this reduction is linked to our finding of an up-regulation of Pde10A. The post-synaptic up-regulation of Pde10A described here argues for a post-synaptic role for the cGMP pathway. Although many reports have concentrated on the functions of cGMP at the presynaptic terminal, some postsynaptic effects of the NO-cGMP pathway have also been described (66, 67, 72).

The Pde10A gene is subjective to alternative splicing (41, 42, 47), and we report the cloning of four novel splice variants Pde10A11–Pde10A14. Although some splice variants may be regulated by protein kinases (47), the function of most splice variants remain to be determined. Apart from a specialized function for each splice variant, it is also possible that the LTP-associated splice isoforms do not possess unique features; the electrical responsiveness of the promotor is then used to generate more protein product. The LTP-associated splice isoforms, Pde10A3, Pde10A6, and Pde10A11, can be expected to exhibit phosphodiesterase activity, as they all contain the diesterase domain. Two GAF domains (for cGMP-binding phosphodiesterases, the cynobacterial *Anabaena* adenylyl cyclase, and the *Escherichia coli* transcriptional regulator fhlA) (73), domains that bind cGMP, are also present in the LTP-associated splice variants.

Our results indicate that some splice variants of Pde10A are up-regulated following the induction of LTP, whereas others (*e.g.* Pde10A2 and Pde10A4) are not. Because all LTP-associated Pde10A splice variants share a common 5′ exon, it is likely that all variants are derived from a common, activity-regulated promotor. Our observations that relative levels of individual splice variants remains similar in different brain regions (as determined by RT-PCR) and the relative levels of Pde10A5/A11 and Pde10A3/A6 splice variants remain similar following LTP induction (as determined by *in situ* hybridization; Fig. 1) argues against the possibility of regulated splicing between LTP-associated Pde10A splice variants.

In summary, using differential display of expressed mRNAs, we have identified two independent transcripts, cl13/19/90 and cl95/96, that are up-regulated following the induction of LTP. We provide evidence demonstrating that these clones are part of the Pde10A primary transcript, suggesting that levels of the primary transcript can be used to study the activity of a gene. Our results indicate that differential display, although primarily aimed to identify changes in expression levels of mRNA, can be used to identify differential expression of other RNA species. Because we and others show that Pde10A encodes a cGMP phosphodiesterase (40–43), our results argue for a role of the cGMP/cGK pathway in long term synaptic plasticity.

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