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, c13/19/90 and c19/96. Neither c13/19/90 nor c19/96 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. c13/19/90 and c19/96 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) 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 isofoms (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 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; cEYFP, enhanced yellow fluorescent protein; cGK, cGMP/cGMP-dependent kinase; EPSP, excitatory postsynaptic potential.

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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 in two independent datasets, c113 and c195/96. 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 unilaterial 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 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 c113/1990 and c195/96 were: Cl19, AGACGCTACATATAGGGC(T)12CA and ACAATTTCACACAGGACTTTCTACCC; Cl95, ACGACTCACTATAGGGC(T)12CA and ACAATTTCACACAGGACTTTCTACCC; and Cl96, ACGACTCACTATAGGGC(T)12CA and ACAATTTCACACAGGACTTTCTACCC. 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 in two independent datasets, c113 and c195/96. 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.

In situ Hybridization—Coronal sections (14 µm) were mounted on polylysine-coated Superfrost slides and stored at −80 °C prior to subsequent analysis by in situ hybridization.

In Vivo 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, 10% dextran sulfate, 500 µM dCTP, 1 ng/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 (30 min), under light halothane anesthesia. Ear-clip electrodes were used to deliver ECS (25). Differential display experiments were performed (25). The primers that led to the differential amplification of c113/1990 and c195/96 were: Cl19, AGACGCTACATATAGGGC(T)12CA and ACAATTTCACACAGGACTTTCTACCC; Cl95, ACGACTCACTATAGGGC(T)12CA and ACAATTTCACACAGGACTTTCTACCC; and Cl96, ACGACTCACTATAGGGC(T)12CA and ACAATTTCACACAGGACTTTCTACCC. 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 in two independent datasets, c113 and c195/96. 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.

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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. 

LTP-associated Expression of Pde10A Splice Variants

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 in two independent datasets, c113 and c195/96. 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.
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.
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. 1B): 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)-carboxyazepan-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. 1C). 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.

c13/19/90 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 c13/19/90 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.
Indeed, identify genes modulated by electrical activity (20, 21, 23, 49). 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. Our observations that (i) specific Pde10A splice variants are up-regulated following LTP induction, (ii) these splice variants have signal intensities of its primary transcript. Indeed, individual Pde10A splice variants had signal intensities -20-fold stronger than those for c13/19/90 and c95/96, 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 c13/19/90 and c95/96, and (iii) expression levels of Pde10A splice variants are markedly higher than those of c13/19/90 and c95/96 favor the hypothesis that c13/19/90 and c95/96 are part of the Pde10A primary transcript.

If c13/19/90 and c95/96 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 c13/19/90 and c95/96. 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 c13/19/90 and c95/96, 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 c13/19/90 and c95/96 have similar expression levels argues against the possibility that c13/19/90 and c95/96 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
common 5' region, which indicates they may be derived from a single promoter. 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 c13/19/90 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–e 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, -A5, 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 TTGTACTGGAGeGATACTTTGGCCG6CA6, with splice sites indicated with 6). 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 promoter. 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 Km 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, c13/19/90 and cl95/96.

Neither c13/19/90 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 c13/19/90 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 A11, 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) c13/19/90 and cl95/96 have an identical expression pattern to the LTP-associated splice variants of Pde10A; (iv) expression levels of c13/19/90 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, c13/19/90 and cl95/96 show other features that can be expected if they are part of a primary transcript: (i) Northern blot analysis using c13/19/90 or cl95/96 failed to identify any full-length transcript, (ii) c13/19/90 and cl95/96 do not contain a large open reading frame, and (iii) c13/19/90 and cl95/96 are themselves intronless.

Our experiments highlight the potential use of differential display to identify changes in expression levels of RNA species.
LTP-associated Expression of Pde10A Splice Variants

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-VIII), 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-c (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, which we coined Pde10A11. The relative levels of individual splice variants were identical in 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. 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-VIII), 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-c (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, which we coined Pde10A11. The relative levels of individual splice variants were identical in 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 part of an intron that is retained in the mRNA. 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 this transcript would form a minor 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 agcgtcgAAAAAAAAAAAAAagagaaaa (5' to 3') for cl13, cl19, and cl90, and gccaagtGAAAAAAGAggAggcegcg for
c95 and c96 (caps denote nucleotides complementary to the RT-primer). These sequences are complementary to the last 14 (cl13 and cl19) and 10 nucleotides (c95 and c96) of the RT primer. The priming site for c90 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 regulatory functions of cGMP at the presynaptic terminal (70); and (iv) brief perfusion of 8-bromo-cGMP before weak tetanic stimulation (Refs. 66 and 68, but see Ref. 69); (iii) a hippocampal stimulation effectors of cGMP include cGMP-dependent protein kinase (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 more protein product. The LTP-associated splice isoforms, Pde10A3, Pde10A6, and Pde10A11, can be expected to exhibit phosphodiesterase activity, as well as contain the diesterase domain. Two GAF domains (for cGMP-binding phosphodiesterases, the cyanobacterial *Anabaena* adenylly cyclase, and *Escherichia coli* transcriptional regulator flhA) (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 promoter. 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, c13/19/90 and c95/96, 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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REFERENCES

LTP-associated Expression of Pde10A Splice Variants