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Dopamine and Glutamate Induce Distinct Striatal Splice Forms of Ania-6, an RNA Polymerase II-Associated Cyclin

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Summary

Control of neuronal gene expression by drugs or neurotransmitters is a critical step in long-term neural plasticity. Here, we show that a gene induced in the striatum by cocaine or direct dopamine stimulation, *ania-6*, is a member of a novel family of cyclins with homology to cyclins K/T/H/C. Further, different types of neurotransmitter stimulation cause selective induction of distinct *ania-6* isoforms, through alternative splicing. The longer Ania-6 protein colocalizes with nuclear speckles and is associated with key elements of the RNA elongation/processing complex, including the hyperphosphorylated form of RNA polymerase II, the splicing factor SC-35, and the p110 PITSLRE cyclin-dependent kinase. Distinct types of neuronal stimulation may therefore differentially modulate nuclear RNA processing, through altered transcription and splicing of *ania-6*.

Introduction

Stimulation of dopamine receptors in the striatum is involved in both normal learning and drug addiction (White, 1997; Schultz, 1998; Berke and Hyman, 2000). Like other forms of long-lasting neural plasticity, these behavioral changes are thought to require at least transient alterations in gene expression (Nguyen et al., 1994; Nestler, 2001). Although many dopamine- and cocaine-induced genes have been identified (e.g., Cole et al., 1992; Brakeman et al., 1997; Berke et al., 1998), the mechanisms by which they affect neuronal function remain poorly understood in most cases. There is evidence that some genes are induced as homeostatic responses to excessive dopaminergic stimulation and that such neuronal adaptations contribute to drug dependence and withdrawal (Shippenberg and Rea, 1997). Recent investigations have started to identify other induced genes that may cause long-lasting changes in behavior by altering synaptic connectivity (reviewed in Berke and Hyman, 2000).

Cyclins and their partner molecules cyclin-dependent kinases (cdks) are now recognized to have important functions beyond regulation of the cell cycle. For example, cdk5 can act as a modulator of dopaminergic signaling in striatal neurons (Bibb et al., 1999). Recently, it has been found in nonneural cells that certain cyclins/cdks can modulate RNA processing in the nucleus through interactions with RNA polymerase II (Pol II). For example, phosphorylation of the Pol II C-terminal domain (CTD) by cyclin T/cdk9 is essential for the transition from initiation of transcription to effective elongation of nascent mRNA transcripts (Peng et al., 1998; Fu et al., 1999).

Here, we describe a novel cyclin, *ania-6*, that is rapidly induced in the adult striatum by cocaine or direct dopamine agonists. The splicing and subcellular targeting of *ania-6* is dynamically controlled by different forms of neuronal stimulation. The longer Ania-6 protein (Ania-6a^{60}) is localized to nuclear speckle compartments; there, it is associated specifically with the hyperphosphorylated form of Pol II (Pol IIo) and other components of the RNA elongation/splicing apparatus, such as the splicing factor SC-35 and the p110 PITSLRE cyclin-dependent kinase. In contrast, the *ania-6* splice variant induced by glutamate stimulation encodes a truncated protein (Ania-6a^{25}) that is not specifically targeted to the nucleus. Different neurotransmitters may therefore cause induction of distinct variants of the same gene. Our observations that neural stimulation can alter levels of a Pol IIo-associated cyclin provide a novel mechanism for regulation of neuronal gene expression and are convergent with multiple lines of evidence suggesting that acute regulation of pre-mRNA splicing is important in neuronal plasticity.

Results

Ania-6 Is a Novel, Inducible Cyclin

An expressed sequenced tag for *ania-6* was previously isolated through differential display PCR (Berke et al., 1998). In adult rats, *ania-6* mRNA is induced in dopamine D1 receptor-expressing striatal neurons following systemic injections of cocaine (Figure 1A) and is intensely induced by a selective D1 receptor agonist in the dopamine-depleted rat model of Parkinson's Disease (Figure 1B). Like other candidate plasticity genes (e.g., Lyford et al., 1995), *ania-6* is also induced in the neuron-like PC12 pheochromocytoma cell line in response to growth factors (Figure 1D). Northern blots for *ania-6* mRNA reveal two major bands, with distinct patterns of baseline expression in different adult tissues (Figure 1C). We used RNA ligation-mediated RACE to obtain full-length cDNA

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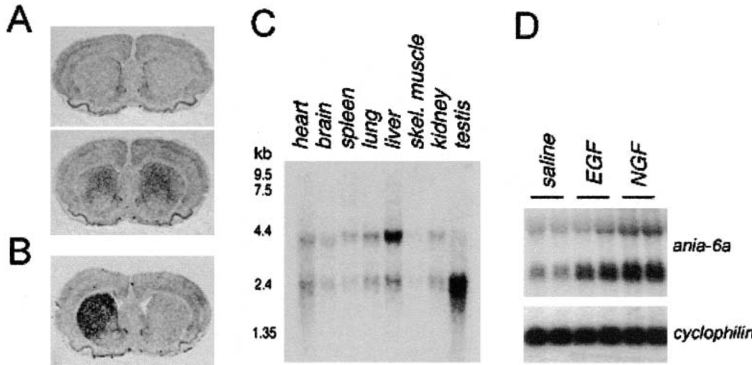

Figure 1. Inducibility of *ania-6a* Transcripts

(A and B) In situ hybridization of coronal brain sections from adult rats, probed for a 5′ *ania-6a* mRNA sequence common to all known splice variants. (A) Animals were killed 1 hr after i.p. injection of saline (upper panel) or cocaine (30 mg/kg) (lower panel). (B) An animal rendered supersensitive to dopamine by a unilateral injection of 6-hydroxydopamine (6-OHDA) into the medial forebrain bundle. The animal was killed 2 hr after i.p. injection of the dopamine D1 receptor agonist SKF38393 (5 mg/kg). 6-OHDA-lesioned side is shown on left. At baseline, there is no side-side difference in expression (Berke et al., 1998).

(C) Multiple-tissue Northern blot (Clontech) showing baseline expression of *ania-6a* transcripts in various adult male rat organs. Very similar results were also obtained with a human multiple-tissue Northern blot (data not shown). Under the stringent hybridization conditions used for all experiments, probes to *ania-6a* and *ania-6b* do not crosshybridize (Figure 4D).

(D) Northern blot of RNA from PC12 cells, 1 hr after administration of saline, epidermal growth factor (EGF, 50 ng/ml), or nerve growth factor (NGF, 50 ng/ml). Upper panel shows expression of *ania-6a* mRNA; lower panel shows same blot after stripping and reprobing for cyclophilin mRNA as control.

clones and found that these two bands correspond to two alternatively spliced mRNAs from the same gene (both mRNAs are induced by cocaine or D1 agonists in vivo; data not shown). The shorter mRNA (~2.1 kb) contains a 1.6 kb open reading frame (ORF) encoding a predicted protein of size 60 kDa. The longer rat mRNA (~4 kb) arises when an exon/intron splice junction is not used (see Figure 3B, below); the resulting additional sequence includes a stop codon, producing a truncated ORF (predicted size 25 kDa; see Figure 2, arrow in panels 2A and 2B). Both predicted proteins contain a region of very high homology to the "cyclin box" that characterizes the many known cyclins (Figure 2B). The longer protein (but not the truncated protein) also contains a C-terminal hydrophilic region (Figure 2C) rich in arginine/serine dipeptides (an RS domain) and four potential nuclear localization signals (Figure 2A).

The expression of *ania-6* mRNA is under very tight temporal control in vivo—following peak induction of *ania-6* in striatum, the mRNA is rapidly degraded, even more rapidly than *c-fos* (Berke et al., 1998). Consistent with this, the 3′ untranslated region common to both *ania-6* isoforms is highly conserved between mammalian species (the last 400 bp of 3′ UTR is 98% identical between human and rat, compared to 93% for the ORF) and contains many AU-rich sequences, including multiple repeats of the pentanucleotide AUUUA (data not shown). Such sequences promote rapid mRNA degradation in various immediate-early genes (IEGs), and loss of these sequences can contribute to oncogenesis (Schiavi et al., 1992).

Inspection of public databases revealed many human, mouse, and rat expressed sequence tags for both of the major *ania-6* mRNA isoforms and also for a closely related but distinct gene. We refer to the *ania-6* gene as "*cyclin ania-6a*" and to the closely related gene as "*cyclin ania-6b*." The tissue distribution of *ania-6b* mRNA strongly resembles that of the larger mRNA form of *ania-6a* (data not shown), and the predicted Ania-6b protein sequence is similarly truncated (Figure 2B). Among previously characterized proteins, Ania-6a and -6b are most similar to those mammalian cyclins (K, T, H, and C) that are part of the RNA elongation/processing machinery (Wei et al., 1998; Edwards et al., 1998). However, the closest protein sequence matches obtained from GenBank were to uncharacterized genes of invertebrate and plant species—for example, a 153 amino acid stretch of mouse Ania-6a is 46% identical to a predicted protein of the plant *Arabidopsis*, while 33% identical to the nearest previously described mammalian cyclin (cyclin K). Ania-6a and -6b therefore represent a distinct, novel cyclin subfamily.

ania-6a Is an Immediate-Early Gene with Independently Regulated Splice Variants

We sequenced mouse genomic DNA for both genes (Figure 3). Consistent with induction by dopamine D1 receptor stimulation, the *ania-6a* promoter contains a "perfect" 8 bp palindromic calcium/cAMP response element (CRE), as well as other regulatory elements that can confer inducible gene expression. We did not identify any consensus sequences for such regulatory elements in the *ania-6b* promoter, and we have not yet found any forms of cellular stimulation that induce *ania-6b* above basal levels (see Figure 4D below). Consistent with the presence of a CRE, *ania-6a* is an IEG in cultured striatal neurons, as *ania-6a* mRNA remains inducible in the presence of protein synthesis inhibitors. In the presence of cycloheximide, *ania-6a* is superinduced by a dopamine D1 receptor agonist, SKF38393 (Figure 4A). Like other IEGs, *ania-6a* expression can be induced by cycloheximide alone; however, this induction was unexpectedly found to be selective for the shorter *ania-6a* mRNA isoform (which encodes the longer protein). To further investigate the regulation of *ania-6a* transcripts, we treated primary cultures of striatal neurons with a variety of stimuli that can induce gene expression (Figures 4B and 4D). Remarkably, we found that each *ania-6a* mRNA can be independently induced. Application of the neurotransmitter dopamine can cause induction of both long and short mRNA isoforms (for quantification, see Figure 4C). In contrast, the neurotransmitter glutamate selectively induces the long mRNA, while a depolarizing concentration of KCl preferentially induces the short mRNA. Different neurotransmitters can therefore

Figure 2. Sequence and Evolutionary Conservation of Ania-6 Protein

(A) Predicted sequence of rat Ania-6a protein. The most highly conserved region ("cyclin box") is shaded; putative nuclear localization signals are outlined. Note also the large number of C-terminal RS dipeptides (an RS domain). Arrow (also in [B]) indicates point of protein truncation predicted for the longer (4 kb) mRNA transcript.

(B) Multiple amino acid sequence alignment for Ania-6a, the closely related Ania-6b, and homologous sequences (ClustalW algorithm, MacVector software). The mouse sequences are given where available to facilitate comparison. Mammalian Ania-6 sequences are closer in sequence to invertebrate homologs than to other mammalian cyclins and closer to cyclins associated with RNA polymerase II (e.g., K, T) than to cyclins directly involved in cell cycle control (e.g., cyclin A1). cDNA and gene sequences for *ania-6a* and *ania-6b* are present in GenBank under accession numbers AF030091, AF159159, AF185590, AF185591, and AF211859.

(C) Conserved C-terminal hydrophilic region, shown using a Kyte-Doolittle plot of hydrophilicity (MacVector). The gray area indicates the area of high sequence identity shown in [B].

induce expression of distinct IEG splice variants in mammalian neurons.

Dopamine induction of both *ania-6a* isoforms likely occurs through the cAMP/protein kinase A (PKA) pathway, since forskolin similarly induces both mRNAs (Figure 4D). Moreover, *ania-6a* induction by dopamine or SKF38393 is blocked by pretreatment with H89, a specific inhibitor of PKA (data not shown). Surprisingly, however, induction of the upper band is selectively suppressed if forskolin is given together with KCl (Figure 4D). Very low concentrations of anisomycin (50 ng/ml) that activate MAP kinase pathways without blocking protein synthesis (Hazzalin et al., 1998) can also induce both short and long mRNA forms, though preferentially the short form.

Distinct *ania-6a* Isoforms Are Targeted to Distinct Cellular Compartments

What might be the function of this selective induction of distinct isoforms by different signal transduction

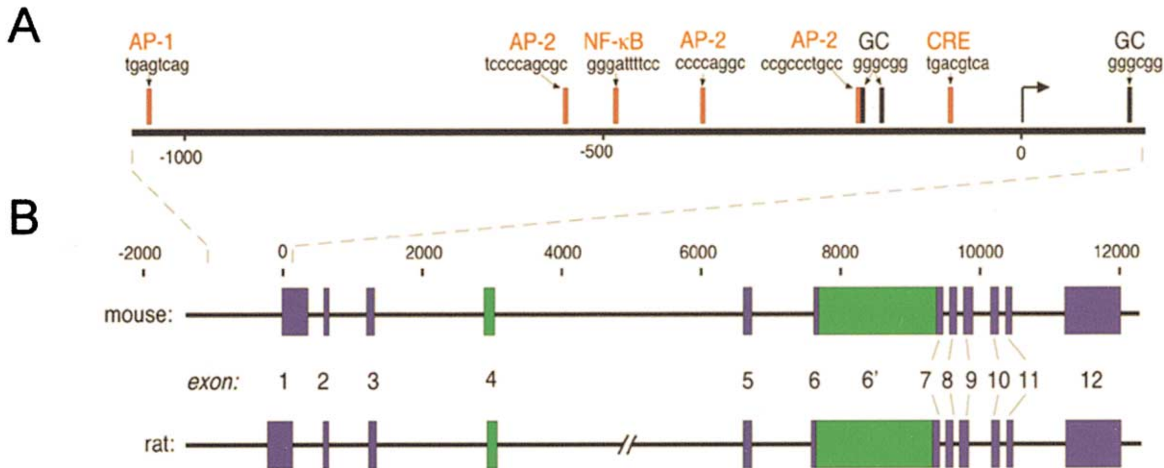

Figure 3. Genomic Structure of the *ania-6a* Gene

(A) Promoter elements identified for mouse *ania-6a*, including an 8 bp palindromic CRE ∼45 bp upstream of the 5′ end of the mRNA. Like some other cyclins and cdks, the *ania-6a* promoter is TATA-less but contains several potential GC boxes; location of the single transcription initiation site was verified by primer extension analysis (data not shown).

(B) Intron/exon structure of the mouse and rat *ania-6a* genes. The region between exons 6 and 7 (shown in green as 6′) was present in some RACE, RT-PCR, and public EST sequences but not others. The 5′ end of this region contains a STOP codon that results in a truncated protein. On rare occasions, we also detected another alternative exon (indicated in green as exon 4) that may result in an even more truncated protein product (Ania-6a^{18}).

pathways? Since the predicted protein sequence of the long protein isoform includes putative nuclear localization signals and an RS domain that are absent in the shorter protein (Figure 2A), one possibility is that the distinct proteins are targeted to different cellular compartments. To test this idea, we generated fusion-protein constructs containing different segments of the *ania-6a* gene and transfected these constructs into PC12 or COS-7 cells (Figures 5 and 6). Fusion proteins that contained either the entire Ania-6a amino acid sequence (Ania-6a^{60}) or just the C-terminal region of the Ania-6a^{60} protein were preferentially localized to the nucleus. In contrast, fusion proteins containing the truncated sequence (Ania-6a^{25}) were evenly distributed to both cytoplasm and nucleus. Differential inclusion of the C-terminal region thus regulates protein localization.

The Long Ania-6 Protein Is Associated with Hyperphosphorylated RNAPolII and Splicing Factors

We noticed that fusion proteins containing the long Ania-6a protein exhibit a "speckled" pattern of expression within the nucleus (excluding nucleoli; Figure 6). In contrast, the truncated Ania-6a protein has a more homogeneous distribution in both nucleus and cytoplasm (data not shown). Nuclear speckles (also called intrachromatin granule clusters [IGCs]) are believed to be storage compartments for nuclear proteins involved in mRNA processing (reviewed in Misteli, 2000). These include the known "SR proteins" that associate with Pol II via its hyperphosphorylated C-terminal domain (CTD) and which (like Ania-6a) contain an RS domain (Caceres et al., 1997). Pol IIo and its associated protein complex are thought to be dynamically recruited from speckles to sites of active transcription (Bregman et al., 1995).

We hypothesized that Ania-6a might also be an RS domain protein associated with Pol IIo and involved in RNA processing. We therefore examined the colocalization of Ania-6a and Pol II (Figures 6A–6H). Using specific antibodies against different forms of the polymerase, we found that the distribution of transiently expressed Ania-6a^{60} corresponds to the distribution of the hyperphosphorylated form of Pol II (Pol IIo) but not to the distribution of the hypophosphorylated form (Pol IIa). Cyclin T1/cdk9 have been recently shown to partially colocalize with Pol II in speckles (Hermann and Mancini, 2001), but, in contrast to cyclin T1, the Ania-6a/Pol II colocalization appears to be both more complete and specific for Pol IIo (compare our Figure 6 to their Figure 3). Using immunoprecipitation techniques, we confirmed that fusion proteins containing the Ania-6a^{60} sequence associate specifically with Pol IIo, not Pol IIa (Figure 7). Although mRNA transcription and processing are tightly coupled in vivo (Neugebauer and Roth, 1997), the hyperphosphorylation of Pol II correlates with its association with splicing factors, not with its transcriptional activity (Kim et al., 1997). Ania-6a fusion proteins also colocalize with a known SR protein and Pol IIo-associated splicing factor, SC-35 (Figures 6I–6L). We have further observed a direct interaction between the Ania-6a^{60} protein and SC-35 in a yeast two-hybrid screen; this interaction was confirmed by a mating test (data not shown).

Ania-6 Is Associated with the "Orphan" Cyclin-Dependent Kinase p110 PITSLRE

In general, cyclins act as modulators of cyclin-dependent kinases. We therefore examined a number of known cdks for possible interactions with Ania-6a. In coimmunoprecipitation experiments, we did not observe any interaction between Ania-6a and cdk5, cdk7, cdk8, or cdk9 (Figures 7G and 7H and data not shown). However,

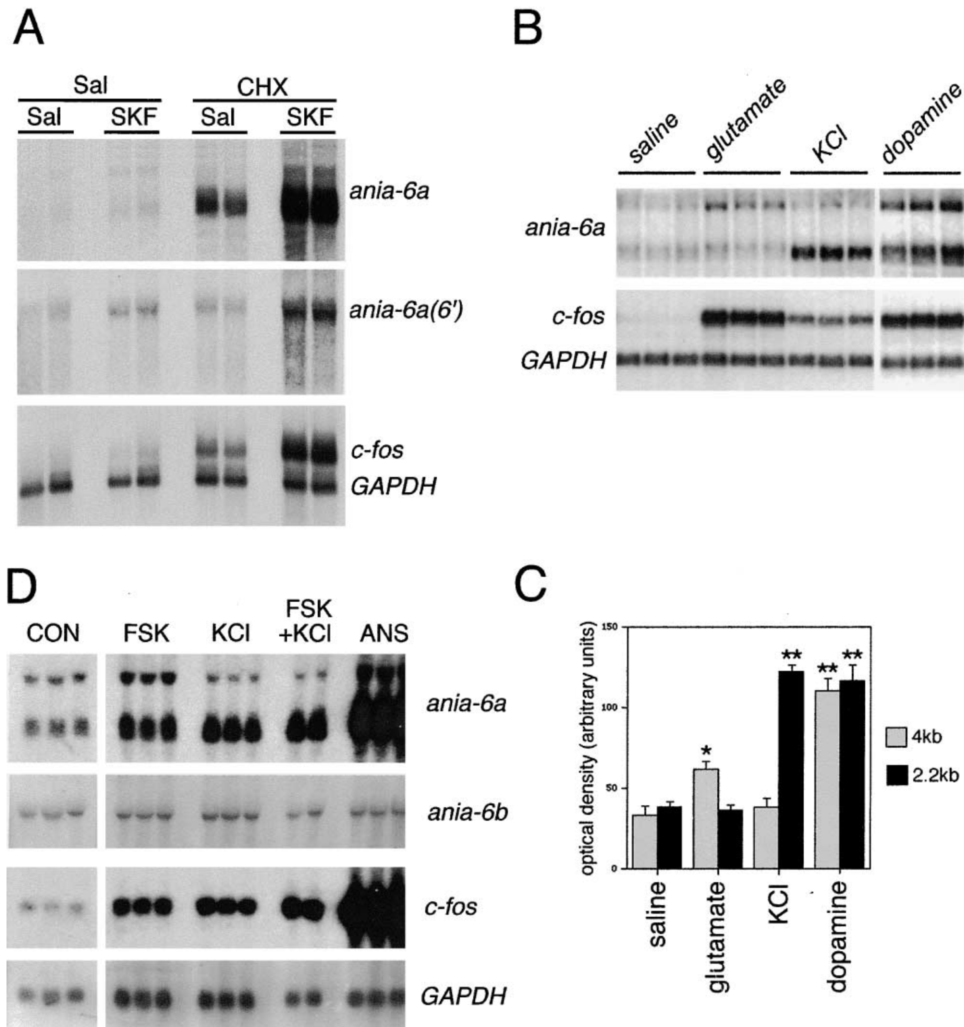

Figure 4. Differential Induction of *ania-6a* mRNA Isoforms

(A, B, and D) Northern blots of RNA from rat primary striatal cultures; each lane represents RNA from an individually treated culture well.

(A) P2 cultures were pretreated with either saline (Sal) or cycloheximide (35 μM, CHX) for 30 min, then treated with either saline or the dopamine D1 agonist SKF38393 (10 μM, SKF) for 90 min. Northern blots were first probed for a 3′ UTR region common to all known *ania-6a* isoforms, then stripped and reprobed for *GAPDH* and *c-fos.* Finally the blot was stripped and reprobed for the alternative exon between exons 6 and 7 indicated in Figure 3B (*ania-6a 6′*), confirming that this sequence is present in the long mRNA form of *ania-6a* but not the short mRNA form. Exposure time for the top panel (*ania-6a)* was shorter (6 hr) than for the middle panel (overnight), to avoid excessive film saturation in the CHX + SKF lanes.

(B) E18 primary striatal cultures were treated with saline, glutamate HCl (100 μM), KCl (50 mM), or dopamine HCl (100 μM), and RNA was extracted 1 hr later. This blot was probed first for *ania-6a* (5′ common region) (upper panel) and then for c-*fos* and *GAPDH* mRNAs (lower panel).

(C) Quantification of representative experiment shown in (B). Histograms show mean ± SEM. “4 kb” and “2.2 kb” refer to the longer and shorter *ania-6a* mRNA variants, respectively. Significance of treatment-induced change was assessed by ANOVA with multiple comparisons (Fisher Protected Least Significant Difference). *$p < 0.01$; **$p < 0.0001$.

(D) *ania-6a* induction in response to KCl, forskolin, and anisomycin. P2 primary striatal cultures were either left untreated (CON) or given a water-soluble analog of forskolin (10 μM, FSK), KCl (50 mM), forskolin and KCl together (FSK + KCl), or anisomycin (50 ng/ml, ANS). RNA was extracted 90 min later. This blot was probed first for *ania-6a* (5′ common region), then for *c-fos* and *GAPDH*, then for *ania-6b*.

we discovered that Ania-6a does interact with another cdk, p110 PITSLRE, and that this interaction is specific for the Ania-6a^{60} protein (Figures 7E and 7F). PITSLRE has several features that are particularly consistent with an interaction with Ania-6a. It has both nuclear and non-nuclear forms, generated by alternative splicing (Xiang et al., 1994) or by differential translation start sites (i.e., IRES utilization; Cornelis et al., 2000). Like Ania-6a, the p110 PITSLRE variant is known to be localized to both nucleoplasm and nuclear speckles (Loyer et al., 1998) and also to interact selectively with Pol IIo, not Pol IIa (Bregman et al., 2000). PITSLRE also contains many of the arginine dipeptide repeats characteristic of spliceosome components, and the p110 isoform is known to interact with the RNA binding protein RNPS1, an activator of mRNA splicing (Loyer et al., 1998; Mayeda et al., 1999). Finally, no cyclin partner for PITSLRE has been previously described.

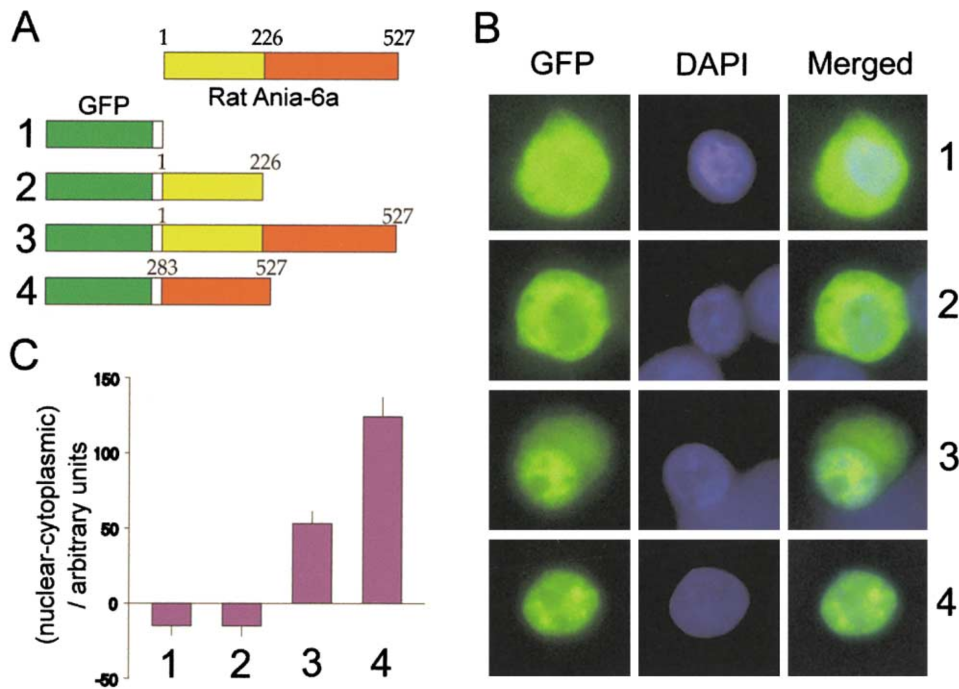

Figure 5. Differential Cellular Localization of Ania-6a Proteins

(A) Structure of GFP fusion proteins (labeled 1 through 4) incorporating distinct portions of rat Ania-6a. Region shown in yellow is common to Ania-6a (both long and short protein isoforms) and also homologous to Ania-6b. Region shown in orange contains the putative nuclear localization signals and the RS domain.

(B) Examples of PC12 cells 8 hr after transfection with the indicated GFP fusion protein, counterstained with the nuclear marker DAPI. Focal plane is through the widest part of the nucleus in each case.

(C) Quantification of GFP data. *y* axis values indicate (in arbitrary units) the intensity of GFP labeling in the nucleus, minus the intensity of labeling in the cytoplasm, after subtracting for background in each case. At least 28 cells were measured for each transfected fusion construct (1 through 4 as in [A]). Statistical comparisons were performed by a one-way ANOVA with all pairwise multiple comparison procedures (Student-Newman-Keuls method). $p < 0.001$ for all comparisons, except when GFP alone is compared with GFP fused to the truncated form of Ania-6a protein (no significant difference).

Our results demonstrate that Ania-6a is a component of the integrated nuclear RNA processing complex that is associated with Pol IIo. Though specifically induced by cocaine or neurotransmitters in the striatum, the widespread tissue distribution of *ania-6* and its strong evolutionary conservation suggest a long-standing important role in nuclear RNA processing. Consistent with this, we have further observed that interference with the *C. elegans* homolog of *ania-6* results in a lethal phenotype (see Supplemental Data at http://www.neuron.org/cgi/content/full/32/2/277/DC1) that closely resembles the effect of interference with Pol II itself or with other essential components of the transcription/splicing machinery (Powell-Coffman et al., 1996).

Discussion

Cyclins and the Adult Brain

The first cyclins were identified and named through their critical function in control of the cell cycle. It is now recognized that cyclins/cdks also have important roles in establishing and maintaining postmitotic differentiated phenotypes, including in neurons (Ross and Risken, 1994; Gao and Zelenka, 1997). In the adult brain, cyclins/cdks have been the subject of recent interest for two major reasons. First, altered expression of cyclins/cdks in response to neurotoxic insults or neurodegenerative disorders has led to the suggestion that apoptotic neuronal cell death can occur through inappropriate reentry into the cell cycle (e.g., Park et al., 1996; Busser et al., 1998). Second, signal transduction pathways involving cdks have been shown to have important functions outside cell cycle control. Cdk5 has received particular attention. It is implicated in the control of the neuronal cytoskeleton and neurite outgrowth, though phosphorylation of microtubule- and actin-associated proteins such as tau (Illenberger et al., 1998; Patrick et al., 1999) and Pak1 (Nikolic et al., 1998). In striatal neurons, cdk5 can phosphorylate DARPP-32 (dopamine- and cyclic AMP-regulated phosphoprotein, 32 kDa), converting it into an inhibitor of PKA activity and thereby reducing the dopamine-stimulated phosphorylation of PKA substrates (Bibb et al., 1999). Cdk5 levels have recently been shown to increase in the striatum in response to chronic cocaine, and this has been suggested to be a neuronal adaptation counteracting excessive stimulation of the PKA pathway by the drug (Bibb et al., 2001). Other cdks are also involved in regulation of the PKA pathway—cyclin B/p34-cdc2 can phosphorylate the type IIβ subunit of PKA and thus prevent the PKA interaction with the anchoring protein neuronal microtubule-

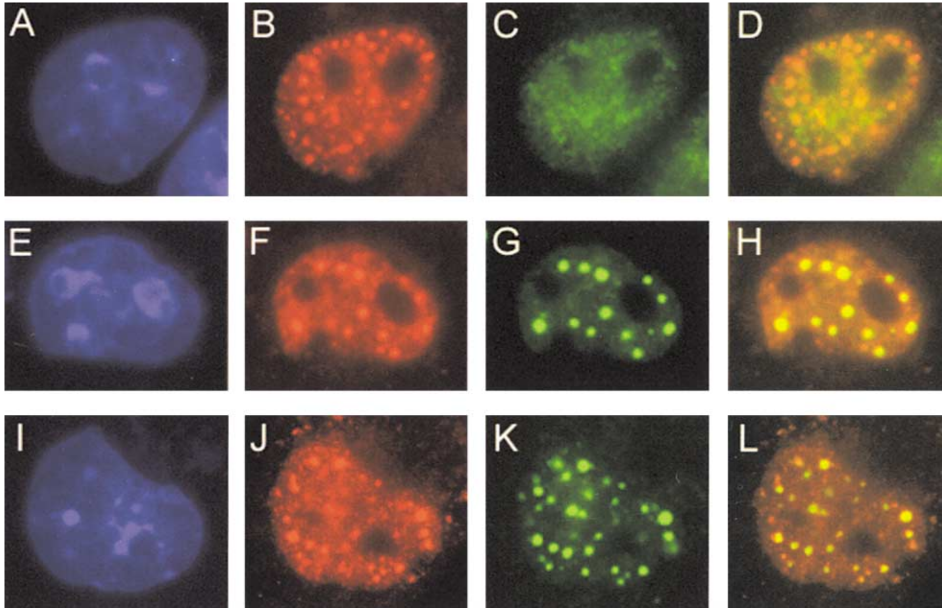

Figure 6. Colocalization of the Longer Ania-6a Protein (Ania-6a[60]) with Hyperphosphorylated RNA Polymerase II and SC-35

(A–L) Representative examples of COS-7 cells transfected with FLAG:Ania-6a[60] and triple labeled using the nuclear stain DAPI (A, E, and I), together with anti-FLAG (B, F, and J), 8WG16 (C), CC-3 (G), or anti-SC-35 (K) antibodies. The CC-3 antibody preferentially recognizes a hyperphosphorylated form of Pol II (Pol IIo), whereas the 8WG16 antibody preferentially binds hypophosphorylated Pol II (Pol IIa) (Vincent et al., 1996). (D), (H), and (L) show the overlay of (B) and (C), (F) and (G), and (J) and (K), respectively.

associated protein 2 (MAP2) (Keryer et al., 1993). Cdks have also been directly implicated in learning processes—the cdk KKIAMRE is induced in the cerebellum of rabbits undergoing eyeblink conditioning but not in those undergoing a pseudoconditioning procedure (Gomi et al., 1999). Our results demonstrate that rapid changes in cyclin expression can occur in the brain in response to abused drugs or neurotransmitters and provide a novel mechanism by which such stimuli may effect changes in gene expression—by altering the composition of the Pol IIo-associated RNA processing complex.

It is now recognized that the various aspects of RNA elongation and processing—such as capping, splicing, and polyadenylation of nascent transcripts—are tightly coupled, interdependent processes (McCracken et al., 1997). Enzymes involved in each of these functions are physically associated with the Pol II CTD, and interference with one aspect (e.g., capping) can disturb another (e.g., splicing) (reviewed in Neugebauer and Roth, 1997). At the present time, our working hypothesis is that Ania-6 is involved in pre-mRNA splicing, given that it posesses a key structural characteristic of SR proteins/spliceosome components (an RS domain) and that it interacts both directly and indirectly with known splicing factors (SC-35, RNPS1). Utilization of alternative splice sites is known to be affected by the phosphorylation state, relative amounts, and subcellular localization of splicing factors (Misteli, 2000). Since Ania-6 is a kinase-associated protein whose levels and cellular localization are modulated by various forms of neuronal stimulation, it is in an excellent position to differentially regulate the splicing of neural genes. However, more work is required to definitively establish the role of Ania-6 in splicing versus other aspects of RNA processing.

Regulated Splicing and Neural Plasticity

Differential splicing is widely used in the nervous system to modify the functional properties and intracellular localization of proteins (e.g., Ramanathan et al., 1999; Okabe et al., 1999; Holmberg et al., 2000). Regulation of alternative splicing can occur in response to nerve injury, neurotoxic insults, or altered stress hormone levels (e.g., Vogelezang et al., 1999; Xie and McCobb, 1998). We have shown in striatal neurons that key neurotransmitters can selectively and rapidly induce distinct mRNA isoforms of the same gene, a mechanism potentially contributing to specificity of genomic responses to neuronal stimulation. Although several neuron-specific RNA binding proteins involved in splicing have been identified (e.g., Koushika et al., 1996; Jensen et al., 2000), the mechanisms underlying neuronal regulation of alternative splicing remain poorly understood in general (Black, 1998). Among protein synthesis-independent mechanisms, it is known that increased intracellular calcium can alter splicing of a Ca-ATPase in a neuroblastoma cell line (Zacharias and Strehler, 1996), and a recent report demonstrated that the altered splicing of BK potassium channels seen following depolarization of pituitary cells involves a CaMKIV-responsive RNA element (Xie and Black, 2001). The cGMP-dependent protein kinase and MKK-p38 signaling pathways have also been implicated in alternative splice site utilization, through phosphorylation or subcellular relocalization of splicing factors (Wang et al., 1999; van der Houven van Oordt et al., 2000). It remains to be determined precisely which mechanisms contribute to differential regulation of *ania-6* splice variants.

In addition to *ania-6*, levels of the splicing factor *transformer-2-*β can be regulated in the rat brain in response

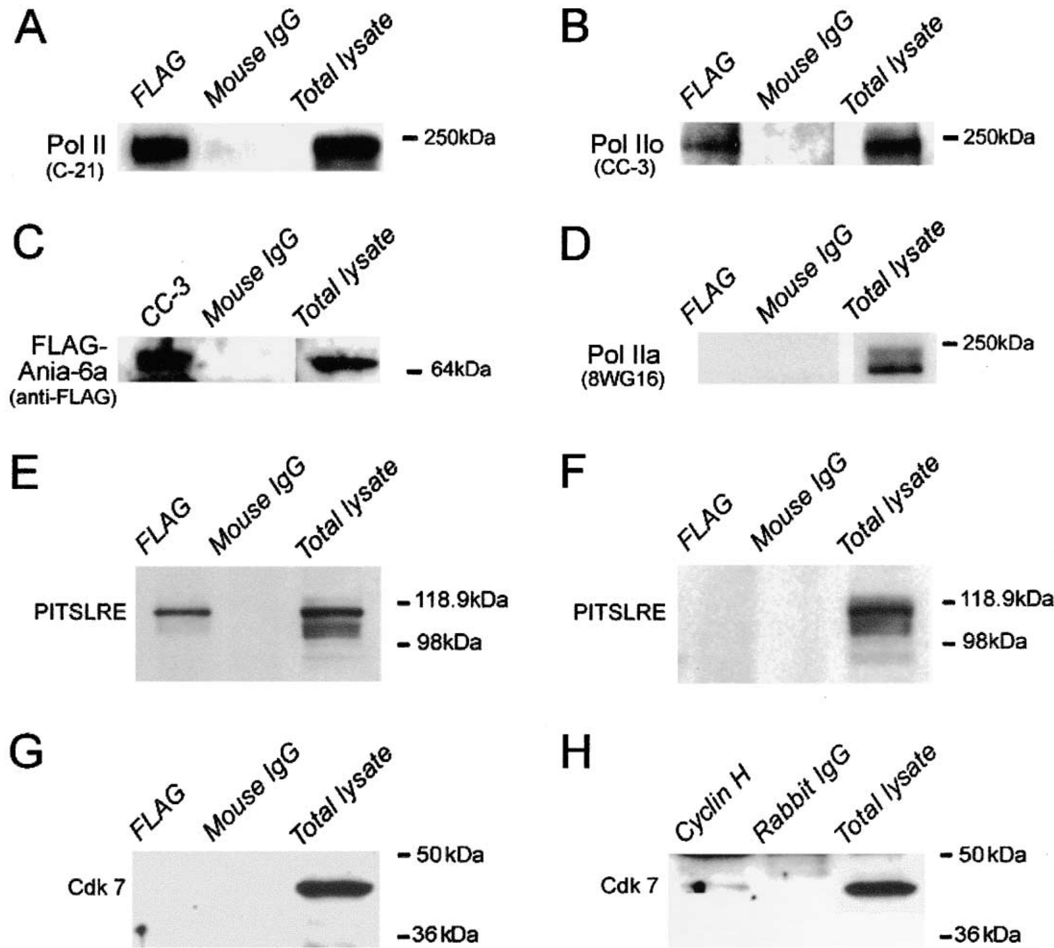

Figure 7. Selective Interaction of Ania-6a with Hyperphosphorylated RNA Polymerase II and the PITSLRE Cyclin-Dependent Kinase

(A–D) COS-7 cells were transfected with FLAG:Ania-6a[60] as in Figure 5. Cell lysates were immunoprecipitated using antibodies against FLAG (A, B, and D), CC-3 (C), or the control mouse IgG and detected using the antibody indicated on the left side of each panel. The C-21 antibody recognizes Pol II but is insensitive to its phosphorylation state. Specificity of other antibodies is described in the Figure 6 legend. (C) The association between FLAG:Ania-6a[60] and Pol IIo is also seen when CC-3 is used for the immunoprecipitation; rabbit IgG is the appropriate control in this case.

(E–H) p110 PITSLRE interacts selectively with the longer Ania-6a protein. COS-7 cells were transfected with FLAG:Ania-6a[60] (E and G) or FLAG:Ania-6a[25] (F). Cell lysates were immunoprecipitated using antibodies against FLAG (E, F, and G) or cyclin H (H), then probed using antibodies against PITSLRE (E and F) or cdk7 (G and H). The cyclin H:cdk7 interaction is used here as a positive control.

to neural activity (Daoud et al., 1999). What neuronal functions might be served by regulating mRNA splicing in response to cocaine or other forms of stimulation? We and others have found that a number of candidate plasticity genes induced by cocaine and dopamine stimulation, including *ania-6*, *ania-4/CaMK-VI*, and *homer-1/ania-3*, have both long and truncated protein isoforms and that the relative proportion of the expressed isoforms is altered by neuronal stimulation (Brakeman et al., 1997; Berke et al., 1998; Vreugdenhil et al., 1999; J.D.B. and S.E.H, unpublished data). Alternative splicing of cell adhesion molecules accompanies long-term synaptic plasticity in *Aplysia* (Schacher et al., 2000), and, in rats, altered splicing of synaptic molecules such as syntaxin-3 is observed in vivo following induction of long-term potentiation in the hippocampus (Rodger et al., 1998). All these observations are consistent with the suggestion that a coordinated activity-dependent change in alternative splicing patterns contributes to neuronal plasticity (Daoud et al., 1999). The functional properties of a number of key striatal molecules, including the dopamine D2 receptor and glutamate NMDA and AMPA receptors, are known to be affected by differential mRNA splicing (e.g., Nakanishi et al., 1992; Usiello et al., 2000). A fuller characterization of neuronal splice regulation may therefore be essential for deciphering the long-lasting responses of striatal neurons to dopamine and other forms of cellular stimulation.

Experimental Procedures

RNA Analysis

All animal procedures were approved by the National Institutes of Health (NIH)/National Institute of Neurological Disorders and Stroke (NINDS) Intramural Animal Care and Use Committee. In all experiments, RNA was extracted using Tri Reagent (Molecular Research). Northern hybridization analyses (2–4 μg of total RNA per lane) were performed using standard methods. Templates for *ania-6* probes were generated by RT-PCR followed by cloning into pCRII-TOPO

(Invitrogen). [^{32}P]UTP-labeled RNA probes were generated by in vitro transcription, using a modified CTP (Strip-EZ; Ambion) to facilitate later blot stripping and reprobing. Hybridization was overnight at 65°C in Ambion NorthernMax hybridization buffer. All RNA blot data shown are representative of at least three separate experiments. An 800 bp region of *ania-6a* 3′ UTR had been previously obtained through differential display PCR (Berke et al., 1998). Primers used to amplify additional probes were as follows. *ania-6a* 5′ common sequence: GGCAGGTGTTGTTTCATCGTTT, GGCAATGGAATTTGA AGTGCTC; *ania-6a* (alternative exon 6′): GGTTTCTCTGCTATTGAG CTGTC, AGCCTGAGCTGGGACTAAATGG; *ania-6b*: AAGGCAGA GAGACGGGTTCTCAAG, CAAGAAGCACAATCGTCAGAGAACCA. Quantification of RNA blots was performed using NIH Image (W. Rasband, NIH). Relative optical density of each band was normalized to GAPDH value for that lane. Unilateral 6-hydroxydopamine lesions and in situ hybridization were performed essentially as described (Berke et al., 1998). Brains from adult male Sprague-Dawley rats (225–300 g) were cut into 12 μm sections, mounted on slides, and hybridized at 55°C with ^{35}S-labeled RNA probes (Riboprobe System, Promega). Slides were exposed for 1 week to 1 month to BioMax MR film (Kodak). RNA ligation-mediated rapid amplification of cDNA ends was also performed essentially as described (Frohman, 1994; Schaefer, 1995). RNA was extracted from the striatum of cocaine-stimulated rats, then dephosphorylated and decapped. A 50 nt RNA oligo (Oligos Etc.) was ligated onto the 5′ end of the mRNA, reverse transcription was performed, and the resulting cDNA used as a template for two rounds (30 cycles each) of high-stringency PCR.

Cloning Genomic DNA

To obtain genomic sequence for both *ania-6a* and *ania-6b*, we first screened a prearrayed mouse ES cell BAC library (Down-to-the-Well, Genome Systems) using PCR. Positive BAC clones (130 kb and 170 kb) were isolated, shotgun libraries constructed, and 1500 sequencing reads performed for each gene, using standard methods. Through contig assembly, we obtained 12 kb of promoter sequence for ania-6a and 27 kb of sequence upstream of the most 5′ identified *ania-6b* exon, at an approximate read depth of 4 to 6X.

Cell Culture, Treatments, and Transfections

Striata were dissected from either E18 rat embryos or P2 rat pups, mechanically dissociated, and plated at 1.5–2 $\times$ 10^6/10 cm^2 well in Neurobasal/B27 medium (Life Technologies) with additional glutamine (0.5 mM), penicillin (50 U/ml), and streptomycin (50 μg/ml). On initial plating, the medium was supplemented with 50 ng/ml BDNF and 30 ng/ml GDNF (Promega). Primary cultures were used at 5–8 days in vitro. Cycloheximide and anisomycin were obtained from Sigma; dopamine HCl, glutamate HCl, SKF38393, and 7β-deacetyl-7β-(γmorpholino)-butyryl-forskolin were obtained from RBI. All drugs were given dissolved in 0.9% saline with 0.001% ascorbic acid. PC12 cells were obtained from the American Type Culture Collection (ATCC) and grown in DMEM-F12 medium, supplemented with 15% horse serum, 2.5% fetal bovine serum, 25 U/ml penicillin, and 25 μg/ml streptomycin (all from Life Technologies). PC12 cells were plated onto poly-D-lysine-coated six-well culture dishes at a density of 10^6 cells/10 cm^2 well. COS-7 cells (obtained from ATCC) were grown in DMEM supplemented with 10% fetal bovine serum, 1% Fungizone (Biofluids), 100 U/ml penicillin, and 100 mg/ml streptomycin (Life Technologies). COS-7 cells were plated on 35 mm or 100 mm tissue culture dishes and transfected at 50% confluency. For PC12 cell transfection, GFP:Ania-6a fusion plasmids were constructed using pcDNA3.1/NT.GFP-TOPO (Invitrogen), and the size of the resulting proteins was checked by in vitro transcription/translation in the presence of [^{35}S]methionine (STP3 system, Novagen, Madison, WI). For transfection of primary striatal cultures, plasmids were coated onto 1 μm gold particles and inserted by biolistic transfection using a GeneGun (BioRad), followed by fixation 8 hours later. COS-7 cells were transfected using FuGene (Roche) with plasmid constructs for FLAG:Ania-6a fusion proteins under the control of an inducible promoter (Ecdysone-Inducible system, Invitrogen). The inducer Ponasterone A (10 μM) was added after 1 day. At the end of the second day, cells were either fixed in 4% paraformaldehyde/phosphate-buffered saline for immunostaining or mechanically lysed in immunoprecipitation buffer (50 mM Tris-HCl [pH 7.4], 100 mM NaCl, 5 mM EDTA, 10 mM NaF, 0.1 mM PMSF, 0.1% NP40, and protease inhibitor cocktail [Roche]).

Immunostaining, Immunoblotting, and Coimmunoprecipitation

For immunostaining, fixed cultures were initially blocked with 10% normal donkey serum/0.5% Triton in phosphate-buffed saline. GFP signal was enhanced using Invitrogen rabbit anti-GFP antibody at a dilution of 1:400, with FITC-coupled anti-rabbit secondary antibody (Jackson ImmunoResearch). FLAG was detected using a goat anti-FLAG antibody (Octa-Probe, Santa Cruz, 1:200 dilution) together with mouse antibodies against Pol II (8WG16, Neoclone, 1:100; CC-3, 1:100) or SC-35 (1:50). Secondary antibodies (1:1000) were Alexa Fluor 568 donkey anti-goat and Alexa Fluor 488 rabbit anti-mouse (Molecular Probes). Cultures were counterstained with DAPI (1:5000; Sigma). Images were captured using a MicroMax 5 MHz cooled-CCD camera (Princeton Instruments) and Metamorph image analysis software (Universal Imaging). Images were quantified by measuring the average density of GFP labeling in a circle of fixed radius, placed either inside the nucleus (as defined by DAPI staining) or in the cytoplasm. Quantification was performed by an investigator blind to the identity of the transfected plasmid. For immunoprecipitation, cellular debris was removed by centrifugation, then lysates were incubated at 4°C for 2–12 hr with antibody (anti-FLAG, Sigma; 8WG16, Neoclone; anti-cyclin H [FL-323], C-21, control mouse IgG, and control rabbit IgG all from Santa Cruz). Protein G-agarose beads (GIBCO-BRL) or protein A-sepharose beads (Pharmacia) were then added for 2 hr at 4°C. After multiple washes, beads were resuspended in 2$\times$ SDS loading buffer and denatured by boiling before electrophoresis through an SDS 8%–16% polyacrylamide gel (Invitrogen). After transfer to nitrocellulose membranes and blocking in TBS-T buffer with 5% milk, primary antibodies were added at the following concentrations: anti-FLAG, 1:1000; CC-3, 1:3000; 8WG16, 1:500; C-21, 1:500; anti-PITSLRE (C17, Santa Cruz), 1:200; anti-cdk7 (FL-346; Santa Cruz), 1:500. After multiple washes in TBS-T, membranes were incubated with appropriate HRP-conjugated secondary antibodies (1:10,000) and washed again prior to visualization using SuperSignal (Pierce) and film exposure (X-OMAT, Kodak).

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Accession Numbers

Sequences reported in this paper have been deposited in GenBank with the following accession numbers: *Rattus norvegicus* cyclin *ania-6a* mRNA, AF030091; *Mus musculus* cyclin *ania-6a* mRNA, AF159159; *Mus musculu*s cyclin *ania-6b* mRNA, AF211859; *Mus musculus* cyclin *ania-6a* genomic DNA, AF185590; *Mus musculus* cyclin *ania-6b* genomic DNA, AF185591.