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EST Analysis of Genes That Are Expressed in the Neural Complex of *Ciona intestinalis* Adults

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ABSTRACT—A subtractive cDNA library was made corresponding to mRNAs expressed in the neural complex relative to those expressed in the pharynx of adults of the ascidian *Ciona intestinalis*. Determination and comparison of expressed sequence tags (ESTs) of a set of 1,527 randomly selected clones demonstrated that they represent 832 independent sequences. Five hundred seventy-two of the clones contained amino-acid-encoding sequences. BLASTX analyses showed that 342 of the 572 clones were strong matches ($P<10^{-7}$) to previously identified proteins, while the remaining 230 fell into the "no match" category. Among the clones matching previously identified proteins, about 80 clones represented proteins that are involved in the formation, maintenance of the structure, and function of the nervous system: 22 proteins are associated with signal transduction, five proteins are related to the synapse, 11 to transcription factors, nine to transporters, five to enzymes, and 13 to extracellular matrix and cytoskeletal components, and six to apoptosis. In addition, sequence information for genes associated with the immune system and for genes encoding proteins with interesting functions were obtained. These data provide cues for further studies on genes that are expressed in and function in the ascidian nervous system.

Key words: ascidians, neural complex, EST analysis, catalog of genes

INTRODUCTION

Ascidians are primitive chordates. *Ciona intestinalis,* a species studied by researchers throughout the world, has a small genome of about 1.6×10^8 bp/haploid, containing approximately 15,500 genes (Simmen *et al.*, 1998). This *Ciona* genome size and gene number are comparable to those of *Drosophila melanogaster* (Adams *et al.*, 2000). This suggests that large-scale cDNA analyses of gene expression profiles would facilitate investigation of the expression and function of developmentally regulated genes during the embryogenesis of this primitive chordate (Satoh, 2001; Satou *et al.*, 2001; Nishikata *et al.*, 2001).

We are interested in genes that are involved in the formation, maintenance of the structures, and function of the central nervous system (CNS) of ascidians (Takamura, 1998; Wada and Satoh, 2001). Ascidians develop two types of CNS in their life: one is formed in the tadpole-type larva and the other in the adult (Katz, 1983; Satoh, 1994; Meinertzhagen and Okamura, 2001). The configuration of the ascidian tadpole is thought to represent the most simplified and primitive chordate body plan (reviewed by Satoh and Jeffery, 1995; Di Gregorio and Levine, 1998), and the larva contains a CNS on the dorsal side of the trunk, which extends as the nerve cord into the tail (Nicol and Meinertzhagen, 1991; Takamura, 1998). The ascidian adults are filter feeders with incurrent and outcurrent siphons, but the tissues and organs constituting the adult show an evolutionary link to those of higher chordates, including vertebrates (e.g., Ogasawara *et al.*, 1999a, b). The CNS of the ascidian adult is usually called the neural complex. The neural complex consists of a small cerebral ganglion and the neural gland, with no known function. The complex lies just dorsal to the anterior end of the pharynx and extends a few nerves to various parts of the body. In the present study we attempted to identify genes that are expressed in the neural complex of *C. intestinalis* adults. Taking advantage of the subtractive hybridization method and ESTs, we were able to identify nearly 80 genes that are associated with the formation, maintenance of the structures, and

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function of the nervous system.

MATERIALS AND METHODS

Biological materials

Adults of *Ciona intestinalis* were collected near Mukaishima Marine Biological Station of Hiroshima University, Hiroshima, Japan. Adults were dissected, and the neural complex and the pharynx were isolated and used for RNA purification.

Construction of subtractive cDNA library

The brain of adult ascidians is called the neural complex, and is composed of the cerebral ganglion and the neural gland. Because the neural complex is closely attached to the pharynx, it is difficult to completely separate this complex from the pharynx. Therefore, we prepared a subtractive cDNA library that contained mRNAs that were more abundant in the neural complex than in the pharynx. Poly(A)$^+$ RNAs were isolated from each organ using a QuickPrep Micro mRNA Extraction Kit (Pharmacia, Piscataway, NJ, USA). Each cDNA library was prepared and subtracted using a PCR-Select^{TM} cDNA Subtraction Kit (Clontech Lab. Inc., Palo Alto, CA, USA) according to the manufacturer's protocol. In brief, cDNAs were synthesized with 2 μg of each poly(A)$^+$ RNA and oligo (dT) primer, and were digested with *Rsa*I restriction enzyme to obtain shorter, blunt-ended molecules. Only digested cDNAs from the neural complex were divided into two fractions, ligated with Adaptors 1 and 2R supplied with the kit, respectively, and hybridized with pharynx cDNAs without adaptors. cDNAs that were not hybridized with pharynx cDNAs were selectively amplified by polymerase chain reaction (PCR) using a primer set annealing to Adaptors 1 and 2R. The subtractive cDNAs were ligated to pBluescript (SK-) phagemid vector (Stratagene) digested with *Sma*I and were electroporated into JM 109 bacteria (TAKARA).

EST sequencing

About 3,500 bacterial clones were randomly picked up from the plates and subjected to plasmid extraction. Templates for sequencing were amplified from these plasmids with M13 forward primer (5'-GTAAAACGACGGCCAGT-3') and reverse primer (5'-GGAAACAGCTATGACCATG-3') by PCR. The sequences of the PCR products were obtained by conventional procedures in an automated ABI 377 sequencer using Big-Dye terminators. The primer for sequencing was M13 forward primer. If the sequence obtained was longer than 500 bp and could not be determined completely, additional sequencing was performed using M13 reverse primer. In this way, we determined the sequences of about 1,500 clones.

Homology search of ESTs

Because we employed *Rsa*I digestion, most of the determined sequences were partial and their average length was about 450 bp (max about 2,000 bp). Thus, these sequences were composed of the UTR (untranslated region) and/or ORF (open reading frame). All sequences were compared with the DNA database (DDBJ/Genbank/EMBL) using the BLAST algorithm (BLASTN and BLASTX). For the sequences containing a putative ORF, their predicted amino acid sequences were also compared with the Protein database (Swissprot) using the BLAST algorithm (BLASTP). On the basis of these results, we categorized these independent sequences into several groups according to function (see Results).

RESULTS

Overall distribution of sequences

In the present study, we made a subtractive cDNA library that contains mRNAs more abundant in the neural complex than in the pharynx of *C. intestinalis* adults. The average length of the cDNAs was about 450 nucleotides. We determined the sequences of a total of 1,527 clones randomly selected from the library. The sequences of most of the clones were determined by reading from one end, and some by reading from both ends. The sequences were compared with one another, and this analysis categorized the 1,527 clones into 832 independent sequences. Because the sequences are partial, the 832 independent sequences do not always represent independent genes. Further sequence analysis revealed that 572 of the 832 cDNAs contained amino acid sequence information of about 120~180 residues each. BLASTX analysis of the 572 cDNAs showed that the amino acid sequences of the polypeptides deduced from 342 of the clones were strong matches ($P<10^{-7}$) to those of previously identified proteins, while the remaining 230 fell into the "no match" category. Of the 342 sequences, 83 clones represent genes that are predominantly expressed in the nervous system, and are listed in Table 1.

Gene catalog

As is evident in Table 1, about 80 genes are associated with the nervous system; they are described below.

(a) Signal transduction: Many signal transduction molecules are responsible for transmission of signals in the nervous system, and therefore it is expected that genes for signal transduction molecules are expressed in the neural complex. The present study identified 22 candidate genes. They include cluster ID 00028, which codes for C3G protein (*P*=5E-61), cluster ID 00034 for cAMP-regulated phosphoprotein (ARPP-19; *P*=5E-61), 00047 for deltex 3, 00069 for protein phosphatase 2C gamma, 00072 for phospholipase A2-activating protein, 00132 for UNC-5 homolog 3, 00133 for protein phosphatase-2A B'epsilon subunit, 00144 for protein phosphatase 2C alpha, 00166 for kappa-type opioid receptor (KOR-1), 00167 for ros-1, 00177 for mitogen activated protein kinase kinase kinase 4, 00168 for Grb2 adaptor protein, 00193 for serine/threonine protein kinase 16, 00212 for RAS-RELATED PROTEIN ORAB-1, 00217 for tyrosine phosphatase-like protein IA-2a, 00228 for a homolog of DPP subclass BMP, 00281 for MARK, 00293 for netrin 4, 00352 for phospholipase D1, 00382 for Toll/IL-1 receptor binding protein MyD88, 00409 for developmental protein tolkin, and 00420 for MAP kinase-interacting serine/threonine kinase 1.

C3G (cluster ID 00028) is a guanine nucleotide exchange factor for Rap1, and C3G-dependent activation of Rap1 is essential for adhesion and spreading of mouse embryonic cells (Ohba *et al.*, 2001). Deltex (cluster ID 00047) is a modulator of neurogenic genes *Notch*, *Delta* and *mastermind* in *Drosophila* (Xu and Artavanis-Tsakonas, 1990). In addition, recent studies suggest that an ascidian BMP is involved in the development of the nervous system (Miya *et al.*, 1997; Darras and Nishida, 2001).

(b) Synapse components: The synapse is connection between the axon and its target cells, and is composed of characteristic proteins. The present study identified cDNA clones for five genes of synapse components: cluster ID 00030

Table 1. Expressed sequence tag similarities, gene description and probability of occurrence by chance

ClusterID	Accession No.	Database Entry Name	Organism	Probability
(a) signal transduction				
00028	BAA04770	C3G protein	*Homo sapiens*	5E-61
00034	AAA30385	cAMP-regulated phosphoprotein (ARPP-19)	*Bos taurus*	6E-10
00047	NP_109639	deltex 3	*Mus musculus*	3E-23
00069	O15355	protein phosphatase 2C gamma	*Homo sapiens*	7E-56
00072	Q9Y263	phospholipase A2-activating protein	*Homo sapiens*	7E-11
00132	NP_033498	UNC-5 homolog 3	*Mus musculus*	4E-81
00133	AAG22076	protein phosphatase-2A B'epsilon subunit	*Xenopus laevis*	1E-41
00144	P49443	protein phosphatase 2C alpha	*Mus musculus*	2E-35
00166	P41144	kappa-type opioid receptor (KOR-1)	*Cavia porcellus*	4E-09
00167	226930	ros-1	*Homo sapiens*	7E-16
00177	NP_036078	mitogen activated protein kinase kinase kinase 4	*Mus musculus*	3E-24
00186	AAB40022	Grb2 adaptor protein	*Mus musculus*	1E-41
00193	P57760	serine/threonine protein kinase 16	*Rattus norvegicus*	4E-24
00212	P22125	RAS-RELATED PROTEIN ORAB-1	*Discopyge ommata*	2E-67
00217	AAA83235	tyrosine phosphatase-like protein IA-2a	*Rattus norvegicus*	4E-50
00228	BAA31132	homolog of DPP subclass BMP	*Halocynthia roretzi*	3E-24
00281	XP_001865	MARK	*Homo sapiens*	4E-18
00293	NP_067295	netrin 4	*Mus musculus*	9E-17
00352	XP_011032	phospholipase D1	*Homo sapiens*	6E-17
00382	AAG10073	Toll/IL-1 receptor binding protein MyD88	*Xenopus laevis*	2E-08
00409	S58984	development protein tolkin	*Drosophila melanogaster*	4E-07
00420	BAA75304	MAP kinase-interacting serine/threonine kinase 1	*Xenopus laevis*	1E-15
(b) synapse				
00030	AAK40302	Tram1	*Xenopus laevis*	1E-21
00064	AAF52148	chr415 synaptotagmin	*Homo sapiens*	1E-34
00185	BAA24980	Ankhzn	*Mus musculus*	1E-31
00296	O42196	cysteine string protein (CSP)	*Xenopus laevis*	1E-22
00424	NP_062172	clathrin heavy chain	*Rattus norvegicus*	2E-60
(c) transcription factors				
00013	BAA02355	Oct-1	*Rattus norvegicus*	1E-26
00085	BAA08722	As-MEF2	*Halocynthia roretzi*	2E-48
00121	CAB42631	msxb homeoproteine	*Ciona intestinalis*	6E-47
00159	AAC60377	bZip transcription factor MafA	*Coturnix japonica*	9E-25
00168	AAC69756	LIM-domain protein	*Branchiostoma floridae*	1E-18
00265	NP_003419	zinc finger protein 84 (HPF2)	*Homo sapiens*	2E-26
00303	NP_005185	CCAAT/enhancer binding protein (C/EBP)	*Homo sapiens*	2E-09
00320	NP_036087	old astrocyte specifically induced substance; BBF-2 (drosophila) homolog	*Mus musculus*	4E-27
00355	S22126	finger protein unkempt	*Drosophila melanogaster*	1E-60
00394	NP_062120	paired-like homeodomain transcription factor 3 (Ptx3)	*Rattus norvegicus*	7E-23
00421	A46050	thyroid/steroid receptor homolog RNR-1	*Rattus norvegicus*	6E-08
(d) transporters				
00001	P30825	high-affinity cationic amino acid transporter-1 (cat-1)	*Homo sapiens*	7E-16
00004	XP_031478	vacuolar protein sorting protein 18	*Homo sapiens*	6E-46
00015	NP_061138	vacuolar protein sorting homolog	*Homo sapiens*	9E-35
00125	AAF76882	dopamine transporter	*Drosophila melanogaster*	2E-13
00135	AAF65254	voltage-dependent anion channel	*Squalus acanthias*	2E-60
00150	P33879	sodium/potassium-transporting ATPase beta-3 chain	*Gallus gallus*	2E-15
00269	AAB97879	NaDC-2	*Xenopus laevis*	5E-30
00413	I38433	excitatory amino acid transporter 3	*Homo sapiens*	9E-19
00418	BAA36692	vacuolar-type H+-ATPase subunit B	*Ascidia sydneiensis samea*	E-107
(e) enzymes				
00056	NP_058769	glutamine synthetase (glutamate-ammonia ligase)	*Rattus norvegicus*	E-118
00084	O02791	galactocerebrosidase	*Macaca mulatta*	5E-10
00145	P15505	glycine dehydrogenase	*Gallus gallus*	6E-68
00288	AAG33131	matrix metalloproteinase 1	*Drosophila melanogaster*	1E-14
00327	XP_007526	aldehyde dehydrogenase 1 family, member A2	*Homo sapiens*	1E-27

(f) extracellular matrix and cytoskeletal components				
00019	AAA28991	tubulin beta	*Drosophila melanogaster*	2E-75
00035	NP_057914	kinesin family member 21A	*Mus musculus*	8E-12
00095	P12716	actin, cytoplasmic	*Pisaster ochraceus*	2E-79
00105	NP_033286	beta fodrin	*Mus musculus*	1E-46
00106	CAC24551	intermediate filament protein B2	*Ciona intestinalis*	E-118
00137	AAC35993	reelin	*Emys orbicularis*	1E-22
00149	AAB41498	alpha II spectrin	*Homo sapiens*	9E-48
00176	AAC39578	tubulin alpha	*Homo sapiens*	3E-77
00215	S23447	annexin XI form B	*Bos taurus*	8E-12
00378	O43405	cochlin precursor	*Homo sapiens*	9E-11
00387	AAK58683	tubulin alpha-1	*Chironomus tentans*	E-123
00397	CAC24554	intermediate filament protein F2	*Ciona intestinalis*	5E-48
00417	AAC27698	actin-filament binding protein Frabin	*Rattus norvegicus*	2E-23
(g) apoptosis				
00063	P42575	caspase-2	*Homo sapiens*	6E-16
00086	CAA71312	eyes absent (eya 1)	*Mus musculus*	1E-63
00136	NP_033818	apoptosis inhibitor 3	*Mus musculus*	2E-15
00195	NP_058021	scavenger receptor class B type I	*Mus musculus*	2E-16
00233	AAB61293	scaffold protein Pbp1 homolog	*Mus musculus*	5E-29
00362	AAD28403	caspase-10/d	*Homo sapiens*	7E-09
(h) immune system				
00115	NP_001542	immunoglobulin-binding protein 1 (alpha 4)	*Homo sapiens*	9E-22
00220	BAA75069	complement C3	*Halocynthia roretzi*	2E-11
(i) ADP-ribosylation				
00161	Q15041	ARL-6 INTERACTING PROTEIN-1 (AIP-1)	*Homo sapiens*	1E-26
00210	NP_062639	ADP-ribosylation-like factor homolog ARL6	*Mus musculus*	3E-50
00405	P51646	ADP-ribosylation factor-like protein 5	*Rattus norvegicus*	4E-37
(j) others				
00002	AAB58114	transformer-2 protein isoform 179	*Drosophila virilis*	3E-15
00075	NP_067286	MIWI protein	*Mus musculus*	1E-21
00117	NP_057525	cysteine-rich motor neuron 1; cysteine-rich repeat-containing protein S52 precursor	*Homo sapiens*	3E-10
00181	NP_079561	superiorcervical ganglia, neural specific 10	*Mus musculus*	9E-08
00307	AAB07067	RNA-binding protein lark	*Drosophila melanogaster*	4E-11
00350	BAB61032	acute morphine dependence related protein 2	*Homo sapiens*	2E-31
00402	BAB40595	Ci-META2	*Ciona intestinalis*	5E-36

codes for Tram1 (*P*=1E-21), 00064 for chr415 synaptotagmin (*P*=1E-34), 000185 for Ankhzn, 00296 for cysteine string protein (CSP) (*P*=1E-22) and 00424 for clathrin heavy chain. A recent study showed that cysteine string protein regulates G protein modulation of N-type calcium channels (Magga *et al.*, 2000), and another showed that NGF signals through TrkA to increase clathrin at the plasma membrane and enhance clathrin-mediated membrane trafficking (Beattie *et al.*, 2000).

(c) Transcription factors: Eleven cDNAs represented genes for transcriptional factors (Table 1). These transcription factors include Oct-1 (cluster ID 00013), As-MEF2 (cluster ID 00085), msxb homeoprotein (00121), bZip transcription factor MafA (00159), LIM-domain protein (00168), zinc finger protein 84 (HPF2) (00265), CCAAT/enhancer binding protein (C/EBP) (00303), old astrocyte specifically induced substance; BBF-2 (*Drosophila*) homolog (00320), finger protein unkempt (00355), paired-like homeodomain transcription factor 3 (Ptx3) (00394), and thyroid/steroid receptor homolog RNR-1 (00421).

(d) Transporter: The present study identified nine cDNA clones for genes that encode transporter proteins. Cluster ID 00001 showed similarity to high-affinity cationic amino acid transporter-1 (cat-1), 00004 to vacuolar protein sorting protein 18, 00015 to vacuolar protein sorting 33B, 00125 to dopamine transporter, 00135 to voltage-dependent anion channel, 00150 to sodium/potassium-transporting ATPase beta-3 chain, 00269 to NaDC-2, 00413 to excitatory amino acid transporter 3, and 00418 to vacuolar-type H+-ATPase subunit B.

(e) Enzymes: It has been shown that many enzymes function predominantly in the nervous system. The present study suggested that five cDNAs were related to such enzymes. Cluster ID 00056 codes for a glutamine synthetase (glutamate-ammonia ligase; *P*=E-118), cluster ID 00084 for galactocerebrosidase (*P*=5E-10), 00145 for glycine dehydrogenase, 00150 for sodium/potassium-transporting ATPase beta-3 chain, 00288 for matrix metalloproteinase 1, and 00327 for aldehyde dehydrogenase 1 family member A2. For example, the mammalian gene for glutamine synthetase is predominantly expressed in asteroglia cells.

(f) Extracellular matrix and cytoskeletal components: Many extracellular matrix and cytoskeletal components play

important roles in the maintenance of the morphology of neuronal cells, formation of axons, and transport of materials through axons. The present analysis demonstrated genes for two types of alpha tubulin (cluster ID 00176 and 00387), beta tubulin (cluster ID 00019; *P*=2E-75), cytoplasmic-type actin (00095), kinesin family member 21A (00035), beta fodrin (00105), intermediate filament protein B2 (00106), reelin (00137), alpha II spectrin (00149), annexin XI form B (00215), cochlin precursor (00378), intermediate filament protein F2 (00397), and actin-filament binding protein Frabin (00417). For example, Miya and Satoh (1997) showed that β-tubulin is expressed specifically in the nervous system of *Halocynthia* larvae. Beta-fodrin is a cytoskeletal protein, and a recent study demonstrated its binding to the merlin-1 product of the NF2 gene (Neill and Crompton, 2001).

(g) Apoptosis: The present study identified six genes that are involved in the process of apoptosis. Clusters 00063, 00086, 00136, 00195, 00233, and 00362 represented genes for caspase-2, eyes absent (eya 1), apoptosis inhibitor 3, scavenger receptor class B type I, scaffold protein Pbp1 homolog, and caspase-10/d, respectively.

(h) Immune-related proteins: Two immune-related genes were identified; one encodes complement C3 (cluster ID 00220) and the other immunogloblin-binding protein 1 (alpha 4) (00115). Ji *et al.* (1997) have characterized complement component C3 from the ascidian *Halocynthia roretzi*, and this report provides similar information about C3 in *C. intestinalis*.

(i) ADP-ribosylation: Three cDNAs were related to ADP-ribosylation. Clusters 00161, 00210, and 00405 showed similarity to ARL-6 INTERACTING PROTEIN-1 (AIP-1), ADP-ribosylation-like factor homolog ARL6, and ADP-ribosylation factor-like protein 5, respectively.

(j) Others: In addition to the genes mentioned above, the present study identified genes for transformer-2 protein isoform 179 (cluster ID 00002; *P*=3E-15), MIWI protein (cluster ID 00075; 1E-21), cysteine-rich motor neuron 1 (cysteine-rich repeat-containing protein S52 precursor) (00117), superiorcervical ganglia (00181), neural specifying, RNA-binding protein lark (00307), acute morphine dependence related protein 2 (00350), and Ci-META2 (00402). *Ci-meta2* was recently identified as a gene whose expression is triggered soon after the initiation of metamorphosis of *Ciona* larvae (Nakayama *et al.*, 2001). The 00402 gene resembles, but is not identical to, *Ci-meta2* (*P*=5E-36).

In addition, although the similarity is comparatively low, BLASTX analysis suggested homologs of several other interesting genes, including those for phospholipase A inhibitor (00074), Notch protein homolog (00114), TANK-binding kinase (00206), Slit (00412), thymosin beta precursor (00103), galanin (00107), gonadotropin-releasing hormone precursor (00139), putative thymosin beta-10 (00214), and mu opioid receptor (00391).

DISCUSSION

In the present study, we performed EST analysis of mRNAs that are preferentially expressed in the neural complex of adults of *C. intestinalis*. We adopted subtractive hybridization of mRNAs of the neural complex minus those of the pharynx to enrich mRNAs of the former in the library. As a result, we identified 22 cDNAs for genes that are associated with signal transduction, five genes for proteins related to the synapse, 11 for transcription factors, nine for transporters, five for enzymes, and 13 for extracellular matrix and cytoskeletal components, and six for apoptosis. In addition, sequence information about genes associated with the immune system and about genes encoding proteins with interesting functions were obtained. These genes are thought to be involved in the formation of the neural complex and its function.

Due to the methods adopted in the present study, the cDNAs characterized in the present study were partial. Therefore, we must determine the complete nucleotide sequences of these clones in future studies. One way to do this is to use the database of *Ciona* cDNA projects. Recently *Ciona* cDNA project consortium members have carried out extensive EST analyses and gene expression profiles of fertilized eggs (Nishikata *et al.*, 2001), cleavage-stage embryos (Fujiwara *et al.*, submitted), tailbud embryos (Satou *et al.*, 2001), larvae (Kusakabe *et al.*, submitted) and young adults (Ogasawara *et al.*, submitted). In collaboration with Academia DNA Sequencing Center of the National Institute of Genetics, Mishima, to date, about 85,000 ESTs have been analyzed and categorized into about 15,000 independent clusters. A preliminary search demonstrated that 61 of the 83 independent sequences identified in the present study show strict matches to those in the *Ciona* cDNA project database. Therefore, taking advantage of this database, we may be able to determine the complete nucleotide sequences of these clones. The sequences of cDNA clones which are not included in the database should be analyzed using the cDNA library of the neural complex.

Strictly speaking, it is rather difficult to isolate only the neural complex from the adult, due to contamination of tissues other than the neural complex. Although we adopted a method of subtractive hybridization to make a cDNA library of the neural complex, it still remains possible that several mRNAs characterized in the present study are expressed in tissues other than the neural complex. This problem is now being addressed by examination of the spatial expression of the genes by whole-mount *in situ* hybridization. It will also be very interesting to ask whether the genes identified in the present study are expressed during the formation of larval CNS.

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