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Short communication

Suppression Subtractive Hybridization Identifies Novel Transcripts in Regenerating *Hydra littoralis*

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Despite considerable interest in the biologic processes of regeneration and stem cell activation, little is known about the genes involved in these transformative events. In a *Hydra littoralis* model of regeneration, we employed a rapid shotgun suppression subtractive hybridization strategy to identify genes that are uniquely expressed in regenerating tissue. With an adaptor-PCR based technique, 16 candidate transcripts were identified, 15 were confirmed unique to mRNA isolated from hydra undergoing regeneration. Of these, 6 were undescribed in GenBank and allied expressed sequence tag (EST) databases (GenBank + EMBL + DDBJ + PDB and the Hydra EST database). BLAST analysis of these sequences identified remarkably similar sequences in anonymous ESTs found in a wide variety of animal species.

Keywords: Differential transcription, Gene, Hydra, Tissue regeneration

Introduction

Hydra represent a remarkably useful model for the study of regeneration, cell-fate determination and patterning largely because of size, simplicity, and rate of regeneration (Steele, 2002; Fujisawa, 2003). The ability of bisected hydra to completely regenerate over a matter of days has been recognized for centuries; however the molecular signals that mediate this remarkable feat are just beginning to be recognized (Technau and Bode, 1999; Hoffmeister-Ullerich, 2001; Frobius *et al*., 2003; Genikhovich, 2006; Thomsen and Bosch, 2006). Studies of hydra have implicated a wide variety of proteins as important in regeneration including members or homologues of the Wnt, DKK, homeobox, insulin receptor, notch and fibroblast growth factor protein families (Grens *et al*., 1996; Martinez *et al*., 1997; Broun *et al*., 1999; Endl *et al*., 1999; Gauchat *et al*., 2000; Reidling *et al*., 2000; Takahashi *et al*., 2000; Cardenas and Salgado, 2003; Broun *et al*., 2005). Numerous signaling pathways and cell-cell communication mediators are active in regenerating or reproducing hydra (Smith *et al*., 1999; Fujisawa, 2004; Sudhop *et al*., 2004). Most of these proteins have been identified by targeted screens or by similarity-of-function tests. The sequencing of the hydra genome is underway and an international team is assembling a hydra EST database (Hydra EST Database). We describe a non-targeted approach to identify expressed sequences unique to a particular developmental or reparative stage, similar to that described by Diatchenko *et al*. (1996). A two-stage subtractive hybridization process was used to rapidly identify 15 expressed sequences found in regenerating hydra.

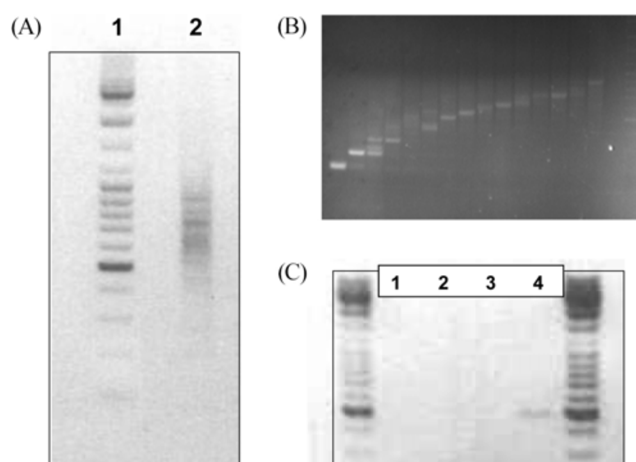

Fig. 1. (A) Electrophoretogram of initial subtracted PCR results: lane 1, marker DNA; lane 2, subtracted PCR mix. (B) Electrophoretogram of laddered individual PCR fragments following gel purification and nested PCR. (C) Representative results of RT-PCR Lanes: 1. Negative control, Unbisected hydra RNA; 2. Unbisected hydra RNA; 3. Negative control, Bisected hydra RNA; 4. Bisected hydra RNA.

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Materials and Methods

Animals and culture conditions. *H. littoralis* were cultured at 21°C under standard conditions (Carolina Biologic Supply Co.). Hydra were fed *Daphnia pulex* daily and were mid-bisected through an axial plane with a scalpel, ten hours after feeding. Head and foot segments were pooled and transferred into fresh medium.

Generation of cDNA and suppressive subtractive hybridization. Poly A+ RNA was isolated from total RNA obtained from both bisected and whole (unbisected) Hydra 18 hours after bisection with NucleoSpin2 RNA Isolation (with DNAse treatment) columns as per manufacturer's protocols (Clontech Inc.). Polyadenylated RNA was converted into cDNA with the Super SMART™ cDNA Synthesis Kit as per standard protocols (Clontech Inc.).

Bisected (tester) and whole (driver) Hydra cDNA was digested with Rsa I to produce short, blunt-ended fragments as per standard protocols (PCR-Select™ cDNA Subtraction Kit, Clontech Inc.). Bisected Hydra cDNA was separated into two aliquots; a unique adaptor (Adaptor 1) was ligated to the 5' end of fragments within one tester aliquot. A second unique adaptor (Adaptor 2R) was ligated to the 5' end of fragments in the second tester aliquot. Each denatured adaptor-ligated tester mix was separately hybridized with single stranded (ss) whole-hydra cDNA driver, to enrich for non-pairing sequences unique to the bisected cDNA pool. A second hybridization included ss driver cDNA as well as the products of the first hybridization from both tester aliquots (adaptor 1 and adaptor 2R linked molecules). In this way, rare molecules, unique to the bisected cDNA pool, could hybridize and form a double-stranded (ds) cDNA flanked by both adaptor 1 and adaptor 2R, thus

Table 1. Non-ordered list describing subtracted cDNA clone BLAST/EST associations, accession numbers, similarity lengths and corresponding E-values (Expectation value refers to the likelihood that a given alignment is expected to occur in a database search by chance; lower scores are more significant.)

Clone/Length	EST Association	Accession No.	Similarity	E-value
1/205	-Hydra ESTs	DT610072	85	$6e^{-20}$
	-*Oncorhynchus mykiss*	CX125585	30	$3e^{-06}$
	-*Zea mays* ESTs	CF054111	35	$8e^{-07}$
	-*Danio rerio* embryonic inner ear cDNA	AL719657	37	$1e^{-05}$
	-Rat liver regeneration (after partial hepatectomy) cDNA	DW398619	57	$5e^{-05}$
2/252	-Hydra ESTs	CK722092	208	$7e^{-55}$
	-*Dictyostelium discoideum* cDNA	BJ375914		0.006
3/266	-Hydra ESTs	CN551222	170	$1e^{-34}$
	-*Triticum aestivum* cDNA	CA739713	61	$5e^{-06}$
	-Rat liver regeneration (after partial hepatectomy) cDNA	DW396890	57	$2e^{-05}$
4/303	-Hydra ESTs	DY450333	177	$1e^{-25}$
5/353	-Hydra ESTs	DT615072	217	$3e^{-69}$
	-*Zea mays* ESTs	CF028547	52	$3e^{-69}$
	-Pine embryo cDNA	CF028547	54	$1e^{-3}$
	-*Triticum aestivum* cDNA	CA735613		$1e^{-6}$
	-*Mus musculus* craniofacial cDNA	AW681411	52	$1e^{-6}$
	-*Enchytraeus japonensis* cDNA	AB236288		$2.3e^{-2}$
6/326	-Hydra ESTs	DT615072	264	$3e^{-131}$
	-*Zea mays* ESTs	CF028547		0.006
	-Rat liver regeneration (after partial hepatectomy) cDNA	DW367938	54	0.006
7/366	-Hydra ESTs	DT620731	32	0.37
8/92	-Human fetal lens	EB461911	25	4.7
	-*Xenopus laevis* cDNA	EB360730	25	4.7
9/150	-*Gasterosteus aculeatus* cDNA	DN734655	19	8.5
10/633	No Association			
11/661	No Association			
12/698	No Association			
13/119	No Association			
14/95	No Association			
15/123	No Association			

forming a unique PCR template. Using a Perkin-Elmer GeneAmp 9600 PCR system, reactions included 27 cycles of denaturation (94ºC, 10 s), hybridization (66ºC, 30 s) and extension (72ºC, 1.5 min). Successive rounds of primary and nested PCR identified distinct cDNA fragments unique to the bisected hydra cDNA mix (Fig. 1). Fragments were gel purified with Qiagen gel purification reagents (Qiagen Inc.) and sequenced by standard techniques (Applied Biosystems) Confirmation that fragments were unique to cDNA from regenerating hydra was obtained by RT-PCR (reverse transcriptase PCR) analysis of bisected mRNA and whole mRNA using fragment-specific sequence for primer design (Fig. 1 and Table 2) with PCR conditions similar to above.

BLAST analysis. All sequences were subjected to BLAST analysis using the NCBI GenBank nucleotide database and other allied public databases (including all available expressed sequence tag (EST) databases) in order to identify similarities (NCBI BLAST).

Results and Discussion

Suppression subtractive hybridization is an excellent strategy to quickly and efficiently identify transcripts that are differentially expressed between two transcriptomes, without prior knowledge of the specific differences. Two rounds of hybridization are sufficient to identify messages present in a tester pool, yet absent in a driver pool. Unique cDNAs can be recovered via PCR, gel purified (Fig. 1) and sequenced. We have demonstrated the power of this approach in the rapid identification of previously unknown transcripts expressed in regenerating hydra, similar to the approach taken by Genikhovich in a study of embryogenesis (Genikhovich *et al*., 2006). Subtractive hybridizations that employed regenerating hydra cDNAs as the test population and whole (non-regenerating) hydra cDNA as the driver identified 16 sequences predicted to be unique to the tester pool. Fifteen of these were confirmed unique by RT-PCR analysis of the original mRNA pools (Table 1). Recovered sequences demonstrate significant similarity to both previously identified Hydra ESTs and genes present in other species. Previously described Hydra ESTs are included in the Hydra EST database as a part of the Hydra EST Project, a collaborative effort aimed at sequencing the ends of 125,000 *Hydra magnipapillata* cDNAs. More than 16,000 distinct EST sequences representing 13,000 different genes have been reported (Hydra EST Database). Cathepsin-L mRNA was identified in our screen; this is well known to be up-regulated during a wide variety of developmental processes including regeneration in Hydra (Hassel *et al*., 1996). Hydra Cathepsin-L mRNA was found to be co-expressed with a variety of protein kinases during hydra regeneration. Cathepsin L up-regulation may permit the processing of a variety of receptor molecules important to differentiation and growth. Interestingly, anonymous sequences were identified in our screen that are similar to those identified in hepatic mRNA pools following partial hepatectomy in *Rattus norvegicus*. Numerous sequences shared similarity with ESTs from *Dictyostelium discoideum*, a species with extensive regenerative capabilities (Bonner, 2003). These similarities are intriguing and suggest possible ancestral regenerative links.

Table 2. Sequence of forward (F) and reverse (R) gene-specific primers

Clone	Primer	Sequence
Clone1:	F Primer:	CCACGAAGCGCTTTCTATGAGTCGG
	R Primer:	CCAGCGCCGGCTCCATGAACAAGCG
Clone 2:	F Primer:	CCTCCCAGTTTTGGGCTGCATTCCC
	R Primer:	CCGAGCTCGCCGGCGGGCCCGTCC
Clone 3:	F Primer:	GGTTTTGTTGACATACCCTCTGG
	R Primer:	CCAGCGCCGGCTCCATGGTATTGG
Clone 4:	F Primer:	CCACAAGTTGGTTCGTAATACACACC
	R Primer:	CCCGTCCATGACAATAATGTTATGCG
Clone 5:	F Primer:	CCCTCCTGGAGATGAGAATGC
	R Primer:	CCAGCGCCGGCTCCATGGTATTGG
Clone 6:	F Primer:	CCACAAGTTGGTTCGAATACACACC
	R Primer:	CCGAGCTCGCCGGCGGGCCCGTCC
Clone 7:	F Primer:	CGAGTGCCTGTACTTCCG
	R Primer:	CCATGGCGTCGTAGCCCGTGACC
Clone 8:	F Primer:	CCAGTCATTAGCTGGTTTATCAGG
	R Primer:	GGGCCCGTCCATGATAAAATGG
Clone 9:	F Primer:	GGAAGGTCACCCCAGCGTGGTTGC
	R Primer:	CCACATGTGTAGCCAAACCCAGCGG
Clone 10:	F Primer:	GGGTCTGCAGAAGCCGTGCGCGG
	R Primer:	GGTCTGGGACTGGCTCCGTGG
Clone 11:	F Primer:	GCTGTCTGCCATGGAACTGTTGG
	R Primer:	GCCGACACGGTCAACGACTACG
Clone 12:	F Primer:	CCAAAGATCTATGAGTCAGGAGG
	R Primer:	CGAAGCCGACACGGTCAACG
Clone 13:	F Primer:	GCTTCGGCTGTGCCAGTTGC
	R Primer:	CGACAGACGGTACCTTGACAACC
Clone 14:	F Primer:	CGTTGGACCAGTATCTGTTGC
	R Primer:	GCAATTTAACTTCTTAATCTGTTAACC
Clone 15:	F Primer:	CGCAGAAGCCGTGCGCGGTGC
	R Primer:	CGCGCCTCGTCGAGAGGTCGTTGG

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