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Lemon Encodes an Unusual Receptor Protein-Tyrosine Kinase Expressed during Gametogenesis in *Hydra*

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In a screen for receptor protein-tyrosine kinase (RTK) genes expressed during gametogenesis in the cnidarian *Hydra vulgaris,* we isolated a cDNA encoding *Lemon,* an RTK with unusual features. *Lemon* is orthologous to *Drosophila* Dtrk, chicken Klg, and human colon carcinoma kinase-4. These genes constitute an RTK class characterized by a conserved transmembrane sequence, the presence of extracellular immunoglobulin-like repeats, and the absence of the DFG motif in the kinase domain. We provide evidence that *Lemon* is a component of an unusual RTK signal transduction mechanism that may involve transmembrane domain-mediated interactions and may not be dependent on its own catalytic activity. *Lemon* transcription is dynamically regulated in interstitial cells during asexual budding and gametogenesis. Transcriptional up-regulation occurs early in spermatogenesis and oogenesis concurrent with the local accumulation of interstitial cells in the body column of sexual polyps.

INTRODUCTION

Extensive morphological and mechanistic diversity exists in the strategies that animals use to generate mature gametes. Because of this diversity, the extent to which molecular gametogenic mechanisms are conserved among multicellular animals is unclear. In order to address this issue, we have been studying gametogenesis in *Hydra vulgaris*, a member of the early-diverging metazoan phylum Cnidaria. The identification and characterization of molecules and molecular interactions that are involved in this elementary system should provide insight into the molecular mechanisms that are fundamental to metazoan gametogenesis.

Gametogenesis in *Hydra* is simple. Sperm- and egg-restricted stem cells derive from interstitial cells located beneath the ectodermal epithelium in the polyp body column (Littlefield, 1985, 1991; Nishimiya-Fujisawa and Sugiyama, 1993). In response to environmental cues that initiate gametogenesis, interstitial cells aggregate and undergo a period of rapid proliferation. Gamete differentiation ensues as the surrounding epithelium develops into a temporary gonad that is used to transfer the gamete(s) to the external medium.

RTKs are membrane-spanning proteins that catalyze intracellular tyrosine phosphorylation reactions in response to extracellular signals (reviewed in van der Geer *et al.*, 1994). These molecular switches are important mediators of developmental signals in many, and perhaps all, animal phyla (Steele *et al.*, 1996). For instance, in mice the Kit RTK and its ligand are essential for germ cell survival, proliferation, migration, and differentiation (reviewed in Bachvarova *et al.*, 1993; Russell, 1979). Thus, RTKs are candidates for molecules involved in regulating gametogenesis in *Hydra*. Using a polymerase chain reaction-based screen, we isolated *Lemon*, a *Hydra* RTK gene with unusual features. We show that *Lemon* is orthologous to chicken Klg (Chou and Hayman, 1991), human colon carcinoma kinase-4 (CCK-4) (Mossie *et al.*, 1995), and *Drosophila* Dtrk (Pulido *et al.*, 1992). These proteins constitute a novel RTK class that is characterized by a conserved transmembrane sequence, the presence of extracellular immunoglobulin-like domains, and the absence of the highly conserved DFG motif in the kinase domain. The aspartate of this motif has been shown to be essential for phosphotransferase activity (Moran *et al.*, 1988; Taylor *et al.*, 1993). We have obtained results which are consistent with the hypothesis that *Lemon* is part of an

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unusual RTK signal transduction mechanism that does not involve kinase activity on the part of *Lemon* and may involve transmembrane domain-mediated interactions, possibly with another RTK. Additionally, we argue that this mechanism has been conserved during animal evolution. An examination of the *Lemon* expression pattern in *Hydra* indicates that this gene has a role in both spermatogenesis and oogenesis.

MATERIALS AND METHODS

Hydra Culture

H. vulgaris polyps were cultured using standard methods (Shimizu and Bode, 1995). Analyses of sexual polyps were carried out using a female strain of *H. vulgaris*, named AEP, which was derived from the PA1 strain isolated by Dr. Carolyn Teragawa (Martin *et al.*, 1997). Males were derived from this strain, which is unstably gonochoristic.

Isolation of cDNA Clones

We used a polymerase chain reaction (PCR) method for the amplification of protein-tyrosine kinase gene fragments (Wilks, 1989; Wilks *et al.*, 1989). The template for amplification was cDNA synthesized from RNA extracted from interstitial cells purified by centrifugal elutriation (Greber *et al.*, 1992), which was generously provided by Martin Greber (University of Munich). The specific amplification conditions have been described previously (Chan *et al.*, 1994). The resulting PCR products were cloned and compared using T-tracking to eliminate fragments from previously identified *Hydra* protein-tyrosine kinase genes. Novel clones were sequenced and analyzed using the BLAST server at NCBI (Altschul *et al.*, 1990; Gish and States, 1993). The 3′ RACE procedure (Frohman *et al.*, 1988) was used to amplify the 3′ end of the *Lemon* cDNA. The resulting fragment was used to isolate a full-length cDNA clone from a Lambda ZAP II cDNA library prepared from adult *H. vulgaris* polyps (Sarras *et al.*, 1994). The methods used for cDNA library plating and screening were essentially as described previously (Bosch *et al.*, 1989; Chan *et al.*, 1994). Inserts from the cDNA clones were recovered in plasmids by *in vivo* excision (Short and Sorge, 1992).

DNA and Amino Acid Sequence Analysis

The sequence of the cloned full-length *Lemon* cDNA was obtained using the Sequenase kit (US Biochemical) and exonuclease III deletions (Henikoff, 1987). Sequence data were compiled and analyzed using DNA Strider (Marck, 1988) and sequence comparisons were performed using the BLAST server at the National Center for Biological Information (Altschul *et al.*, 1990; Gish and States, 1993). Phylogenetic analysis was performed using maximum parsimony methods. Kinase domain amino acid sequences from a diverse sampling of RTKs were used in the analysis. The presence or absence of extracellular immunoglobulin-like domains, kringle domains, EGF-like repeats, fibronectin III domains, leucine-rich motifs, and discoidin-1-like domains was also used in the matrix. The heuristic search option of PAUP* 3.1 (Swofford, 1993) was used for tree construction, with 200 random order taxon addition replicates and tree bisection and reconnection branch swapping. Human Src, a non-receptor protein-tyrosine kinase, was used as the outgroup. The "protpars" matrix of PAUP* 3.1 was used to weigh amino acid substitutions. To obtain bootstrap values, 100 bootstrap replicates were performed using simple taxon addition with tree bisection and reconnection branch swapping. Homology models of protein structures were created using SWISS-MODEL (Guex and Peitsch, 1997; Peitsch, 1995), available through the ExPASy WWW molecular biology server from the Swiss Institute of Bioinformatics (Appel *et al.*, 1994). Model evaluations using the Profiles 3-D program (Luthy *et al.*, 1992) and Prosa (Sippl, 1993) were provided with the returned model. Model evaluations using Whatif V4.99 (Hooft *et al.*, 1996; Rodriguez *et al.*, 1998) and Procheck V3.5 (Laskowski *et al.*, 1993; Morris *et al.*, 1992) were performed using the Biotech Validation Suite for Protein Structures at http://biotech.embl-heidelberg.de:8400.

In Situ Hybridization

In situ hybridization with whole-mount preparations was performed as previously described (Martínez *et al.*, 1997). Digoxigenin-labeled sense and antisense RNA probes were generated by *in vitro* transcription of a fragment from the region of the *Lemon* cDNA encoding the extracellular domain. Samples were photographed with Nomarski optics after permanent mounting in Euparal (Asco Laboratories). For the identification of individual interstitial cells expressing *Lemon*, testes- or egg-forming regions were dissected and dissociated into individual cells using the maceration technique (David, 1973). Cells were then fixed in 4% paraformaldehyde for 30 min and spread on subbed glass slides. *In situ* hybridization to the dispersed cells was performed as described by Kurz *et al.* (1991) except that solutions and detection methods were identical to those in the whole-mount *in situ* hybridization procedure of Martínez *et al.* (1997). Hybridization was carried out at 55°C for 36 h in a sealed chamber surrounding the cells. Posthybridization washes were done at 55°C in 2× SSC plus 0.1% CHAPS four times for 30 min each in a Coplin jar. Cells were then mounted in PBS:glycerol (9:1) and photographed with Nomarski optics.

RNA Hybridization Analysis

Poly(A)$^+$ RNA from adult *H. vulgaris* polyps was generously provided by Dr. Andy Shenk (UC Irvine). Electrophoresis of the RNA, transfer to nylon membrane, hybridization, and posthybridization washes were done as previously described (Shenk *et al.*, 1993).

Bromodeoxyuridine Labeling

To identify cells that were in S phase of the cell cycle, polyps were injected in the gastric cavity with 5.0 mM 5-bromo-2′-deoxyuridine (BrdU) from Sigma (Plickert and Kroiher, 1988). One hour later, animals were relaxed in 2% urethane and fixed in 100% ethanol. Samples were incubated in 2 N HCl for 30 min, rinsed in PBS four times, and incubated with an anti-BrdU antibody (Becton–Dickinson). A fluorescein-conjugated secondary antibody was used to detect the bound anti-BrdU antibody. Preparations were mounted in 9:1 PBS:glycerol and photographed.

Elimination of Interstitial Cells by Hydroxyurea Treatment

The number of interstitial cells was reduced by treating polyps with 10 mM hydroxyurea for either 1 or 3 days (Sacks and Davis,

1979). After a 2-day recovery period, several polyps were macerated (David, 1973) and the ratio of large interstitial cells to epithelial cells was determined.

Protein Expression in Yeast

Fragments of genes of interest were amplified using PCR with gene-specific primers flanked by the appropriate restriction enzyme cleavage sites for cloning. All site-directed mutations were created using standard PCR strategies (Higuchi *et al.*, 1988; Vallette *et al.*, 1989) and verified by sequencing. The amplified fragments were isolated from the reaction mixtures with QIAEX particles (Qiagen), cleaved with appropriate restriction enzymes, and purified by agarose gel electrophoresis. Fragments were ligated into the galactose-inducible yeast expression vector pRS316-GAL1 (Liu *et al.*, 1992). Strain W303 of *Saccharomyces cerevisiae* was transformed with plasmid DNA using the lithium acetate method. Transformants were selected on minimal medium lacking uracil. For expression of proteins, plasmid-containing yeast cells were grown at 30°C in uracil-minus minimal medium plus glucose overnight and then diluted in uracil-minus minimal medium plus galactose and grown for an additional 12 to 20 h. Cells were harvested by centrifugation and proteins were extracted (Yaffe and Schatz, 1984). Protein concentrations were determined using the BCA Protein Assay Reagent Kit (Pierce). Equal amounts of protein were fractionated by SDS–PAGE and transferred to an Immobilon-P filter (Millipore) using a Bio-Rad Trans-Blot SD semidry electrophoretic transfer cell. For the anti-phosphotyrosine blots, the filter was blocked in 5% BSA for 1 h at room temperature and incubated with 4G10 anti-phosphotyrosine monoclonal antibody (Upstate Biotechnology) diluted 1:13,000 in Tris-buffered saline plus 0.05% Tween 20 (TBST). For the anti-myc blots, the filter was blocked in 5% nonfat dry milk for 1 h and incubated with the 9E10 anti-myc monoclonal antibody (Santa Cruz Biotechnology) diluted 1:200 in TBST plus 5% nonfat dry milk. The filter was then washed at room temperature five times for 5 min each in TBST. Following incubation for 1 h at room temperature with a horseradish peroxidase-conjugated goat anti-mouse IgG antibody (Transduction Laboratories) diluted 1:25,000 in TBST, the filter was washed at room temperature four times for 5 min each in TBST. Bound antibody was detected with the SuperSignal chemiluminescent substrate for Western blotting (Pierce).

A

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  1 MFLPLIYKIVQWFLVILLCNNDLIQNITTAVTVIVEPRNVLQTAKTEVFVNCTTSPVEDA
 61 QIIWNKGEENIPSYSEGTKDEVYVLPNNTLYLKSLKKKEAGTYPCNAFLTLEKLFQTVVI
121 EVAFLQKLNSIDPVKVLQGYVAEIVCEEPVGKPQPKVKWLYNGATISPDNPNIDSSSWTL
181 RIKKTMLSDAGNYTCVAFNSVAERTAEAKLTVVVVGDVDVSPISNSIKEGESATFKCESK
241 SDPPLNVKWKRDIETCSTTDNSQSVCKVTKEDVIPSDRVIISGGTLSIKNASVKDEGIYL
301 CETELSTQISSAKVQLNVFELMKVDKSLHNEIQTCDRLRQNNTKITCHFRGDGKYSIQWT
361 RLSPNAILNSNMKVVNDSIYISELKFEDMGQPKCEAVGEYNNATAYVNFIVYEHPQFIIS
421 PKNITAYIGEPAWVHCQGKGFPKPRVYILRGKKDGGSLNNSYFVQLPNDTFYIKSLKKEY
481 DGEYFCWLIEQYGSISDTFSITVLEKEVNTGKGSPMGRTVGIAVGCAGVYILLVIGLMIY
541 CRARRARLFKKGKLVEVDGEPLAEDHLLGASDIPLVSIEKLIFNYENLQEIIVLGYGKFG
601 KVFKAYAKGICEEGTDTLVAVKVFEETPVNNVLSAFNKETEDLMQFQNSYVVRLLGLVRE
661 SPFCIITEYSELGDLKEYLQTNKNTSSSARLHMCTCIAKGMSYLATLHYVHRDLAARNCI
721 LFSQNEVKVSFLSLCNTTYKDDYYLLNNLLAPVRWLAPESIRENIYNEKTDVWSFSVTMW
781 EIFSSEVQPFYGVSNEEVVNRIGKDLQLTIPSNCPKTLYKIMTRCWLVNAFDRPTFDELL
841 SLMAEISH
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B

Most RTKs	DFG
Lemon	FLS
Klg	ALS
CCK-4	ALG
Dtrk	YPA

C

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Lemon      SMGRTVGIAVGCAGVYILLVIGLMIYCRARR
Klg        KMIQTIGLSVGAAVAYIIIVLGLMFYCKKRR
CCK-4      KMIQTIGLSVGAAVAYIIAVLGLMFYCKKRC
Dtrk       LVTRAVLITMTVALAYIVLVVGLMLWCRYRR
consensus      T G  VG A AYI  V GLM YC
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FIG. 1. (A) Predicted amino acid sequence of the *Lemon* protein. The predicted transmembrane sequence is indicated by single underlining. Bold-faced residues indicate amino acid positions identified by Hanks and Quinn (1991) that are highly conserved among protein-tyrosine kinases. Bold-faced residues that are doubly underlined are not conserved in *Lemon*. (B) Amino acid sequences replacing the DFG motif in the kinase domain of *Lemon* and its orthologues. (C) Alignment of the transmembrane sequences of *Lemon* and its orthologues. The transmembrane sequences are underlined. Residues present in at least three of the four sequences are shown on the consensus line.

RESULTS

Identification and Cloning of the Lemon Gene

We used a method originally developed by Wilks *et al.* (1989) to isolate genes encoding RTKs expressed in interstitial cells. The kinase domain amino acid sequence encoded by one cloned fragment (*Lemon*) showed significant sequence identity to the kinase domain of the chicken Klg gene product (Chou and Hayman, 1991), the human CCK-4 gene product (Mossie *et al.*, 1995), and the *Drosophila* Dtrk gene product (Pulido *et al.*, 1992). A 3.4-kb cDNA clone for *Lemon* was found to contain an open reading frame of 2550 bp that encodes a predicted protein product of 848 amino acids (Fig. 1A). A potential initiator methionine is followed by a stretch of hydrophobic amino acids that is characteristic of a signal peptide sequence. The predicted protein contains five immunoglobulin-like domains followed by a transmembrane sequence and a protein-tyrosine kinase catalytic domain. Hybridization of a fragment from one of the *Lemon* cDNA clones to poly(A)$^+$ RNA from nonsexual adult polyps detected a single 3.4-kb RNA species (data not shown), the same size as the cDNA clone which was sequenced. Taken together, these results indicate that we have cloned the entire coding region of the *Lemon* gene and that the gene encodes a receptor protein-tyrosine kinase.

Lemon Encodes an Orthologue of Dtrk, Klg, and CCK-4

The *Lemon* kinase domain is unusual because a highly conserved sequence motif that has been shown to be

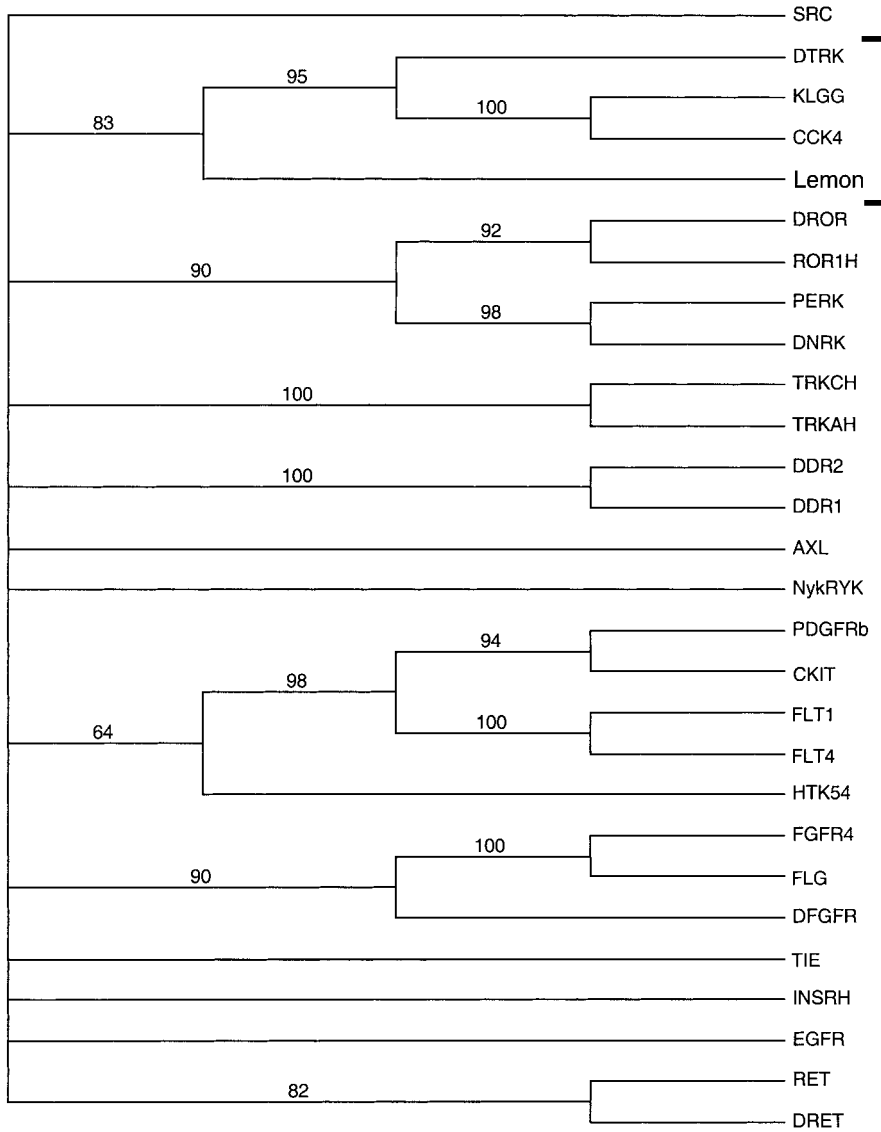


FIG. 2. Results from maximum parsimony phylogenetic analysis of selected RTKs. Bootstrap values are shown above the branches. A score of 80 or higher indicates very strong support for that particular branch. *Lemon* and its orthologues are indicated by the bracket. See Materials and Methods for details of the analysis. DTRK (S19247), DNRK (AAD02091), DROR (A48289), DFGFR (Q09147), and DRET (BAA21836) are from *Drosophila melanogaster;* INSRH (P06213), TRKAH (P04629), TRKCH (Q16288), SRC (P00523), ROR1H (A45082), DDR1 (Q08345), DDR2 (AAA18019), AXL (P30530), PDGFRb (P09619), FLT1 (P17948), FLT4 (P35916), FGFR4 (P22455), FLG (P11362), EGFR (P00533), RET (P07949), and CCK-4 (AAA87565) are from *Homo sapiens;* PERK (AAA49285) is from *Torpedo californica;* KLG (A39712) and CKIT (Q08156) are from *Gallus gallus;* NykRYK (Q01887) and TIE (Q06806) are from *Mus musculus;* and HTK54 (AAA65223) and LEMON (AAB03389) are from *Hydra vulgaris.* The Entrez accession numbers for the protein sequences are in parentheses.

essential for catalytic activity is altered. Three other RTKs (Klg, Dtrk, and CCK-4) possess similar alterations (Fig. 1B) and a BLAST search with the *Lemon* kinase domain sequence gave results which ranked these three proteins highest in amino acid sequence identity scores. In order to determine the phylogenetic relationship of these four unusual proteins within the RTK family, we performed a maximum parsimony analysis using kinase domain amino acid sequences and the presence or absence of several characteristic RTK features (Hanks and Quinn, 1991). The results of this analysis (Fig. 2) strongly support the hypothesis that *Lemon,* Klg, Dtrk, and CCK-4 are orthologues.

Lemon, Klg, CCK-4, and Dtrk thus constitute a class of RTKs characterized by the alteration of the DFG motif

within the kinase domain and the presence of extracellular immunoglobulin-like repeats. A third feature of this class, which was not used in our phylogenetic analysis, is a highly conserved transmembrane sequence. The *Lemon* transmembrane sequence is 55% identical to the transmembrane sequences of Klg and CCK-4 (Fig. 1C). This degree of transmembrane sequence conservation across such a large evolutionary distance is apparently unique among known RTKs.

Lemon Does Not Phosphorylate Itself or Yeast Proteins in Vivo

Mutagenesis studies have demonstrated that the DFG motif is essential for kinase activity (Katso *et al.,* 1999; Moran *et al.,* 1988; Taylor *et al.,* 1993). *Lemon,* Dtrk, Klg, and CCK-4 have alterations in this DFG motif (Fig. 1B). To test the *Lemon* catalytic domain for protein-tyrosine kinase activity, we took advantage of the fact that the yeast *S. cerevisiae* contains a very low level of proteins phosphorylated on tyrosines and no canonical protein-tyrosine kinases (Hunter and Plowman, 1997). The *Lemon* kinase domain was cloned into a galactose-inducible vector for protein expression in yeast. In addition to this construct, two positive control constructs and one negative control construct (vector alone) were tested. Our positive control constructs encoded the catalytic domain of *Sweet Tooth,* a *Hydra* RTK possessing a typical catalytic domain (Reidling *et al.,* 2000), and *Xenopus* Src (Steele *et al.,* 1989). Transformed yeast cells were grown in the presence of glucose or galactose for 20 h. Total protein extracts were fractionated by SDS–PAGE, transferred to a membrane, and probed with an anti-phosphotyrosine antibody. As shown in Fig. 3, protein extracts from strains containing *Sweet Tooth* and Src grown in galactose possess large numbers of proteins phosphorylated on tyrosine. In contrast, an extract from yeast cells producing the *Lemon* kinase domain contained the same pattern of tyrosine-phosphorylated proteins as a negative control extract. Synthesis of the *Lemon* protein in the yeast cells was verified by attaching a myc epitope tag to the carboxyl terminus (Fig. 3). These results demonstrate that the *Lemon* catalytic domain is not functional in yeast under conditions under which catalytic domains from other diverse protein-tyrosine kinases are functional. We cannot rule out the possibility that the *Lemon* kinase domain has an unusual substrate specificity, such that it does not recognize any yeast protein. However, the fact that it does not even autophosphorylate in yeast cells argues that it is catalytically inactive.

Ryk, an RTK which is not closely related to *Lemon,* also lacks kinase activity and contains the sequence DNA in place of the DFG motif (Hovens *et al.,* 1992; Katso *et al.,* 1999). Upon replacement of the DNA sequence with DFG by site-directed mutagenesis, Ryk acquires kinase activity (Katso *et al.,* 1999). We used site-directed mutagenesis to change the FLS sequence present in the *Lemon* kinase domain back to the ancestral sequence DFG in order to see if we could restore *Lemon* kinase activity. When this mutated kinase domain was expressed in yeast, no increase in the level of tyrosine-phosphorylated proteins was observed relative to negative controls (Fig. 3, lane 12). This result raises the possibility that some aspect of the structure of the *Lemon* kinase domain deviates from known protein-tyrosine kinase domain structures despite the fact that 32 of the 40 most conserved kinase domain amino acids (Hanks *et al.,* 1988) are present (Fig. 1A). However, homology modeling of the *Lemon* kinase domain and subsequent systematic comparisons of the model's conformational, energetic, environmental, and packing properties with the corresponding properties of known RTK kinase domain structures (data not shown) indicate that the *Lemon* kinase domain is not likely to deviate significantly in structure from other RTKs. Similar results were obtained when homology models of Klg and CCK-4 were created and evaluated (data not shown). These results indicate that the structure of the kinase domain of *Lemon* family RTKs has been conserved over the course of most of metazoan evolution despite the presumed absence of catalytic activity throughout that period.

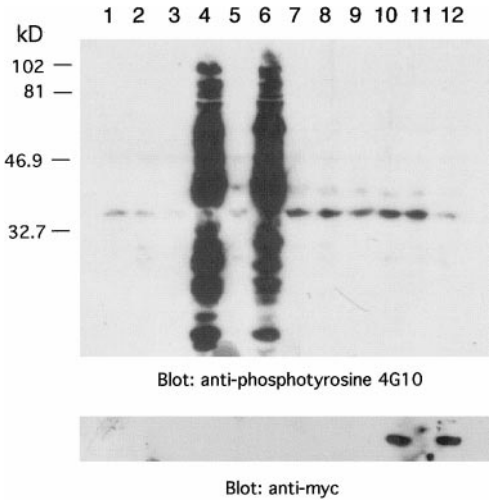


FIG. 3. Detection of phosphotyrosine-containing proteins in yeast cells expressing various *Hydra* protein-tyrosine kinase catalytic domains. *S. cerevisiae* cells were transformed with various constructs under the control of a galactose-inducible promoter (see Materials and Methods) and grown in the presence of glucose or galactose. Extracts containing equal amounts of protein were fractionated using SDS–PAGE, blotted, and probed with the indicated antibodies. (1) Vector alone, glucose; (2) vector alone, galactose; (3) *Xenopus* Src2, glucose; (4) *Xenopus* Src2, galactose; (5) *Sweet Tooth* kinase domain, glucose; (6) *Sweet Tooth* kinase domain, galactose; (7) *Lemon* kinase domain, glucose; (8) *Lemon* kinase domain, galactose; (9) *Lemon* kinase domain-myc, glucose; (10) *Lemon* kinase domain-myc, galactose; (11) *Lemon* kinase domain FLS → DFG-myc, glucose; (12) *Lemon* kinase domain FLS → DFG-myc, galactose.

The Lemon Transmembrane Sequence Affects Protein Stability

The *Lemon* transmembrane sequence has been highly conserved during evolution, suggesting that this sequence is important for proper protein function (Fig. 1C). Although this degree of transmembrane sequence conservation is apparently unique among RTKs, it is common in membrane-spanning multisubunit protein complexes and in many integral membrane proteins which cross the bilayer more than once (reviewed in Harrison, 1996). Specific interactions between transmembrane α-helices are important for the assembly of receptor complexes such as the T-cell antigen receptor, the B-cell receptor, CD8, FcγRI, glycophorin, and MHC class II molecules (Blum *et al.*, 1993; Bonifacino *et al.*, 1990a,b; Cosson and Bonifacino, 1992; Harrison *et al.*, 1995; Hennecke and Cosson, 1993; MacKenzie *et al.*, 1997).

To investigate possible roles of the *Lemon* transmembrane sequence, myc epitope-tagged recombinant proteins derived from the *Lemon* gene or from two other *Hydra* RTKs were produced in yeast under control of the GAL promoter. The two other RTKs were HTK7, the *Hydra* homologue of the vertebrate insulin receptor (Steele *et al.*, 1996), and HTK54 (Chen and Steele, unpublished results), a member of the PDGF receptor family (Fig. 2). The protein produced by each construct possessed the *Lemon* signal sequence. In addition, the *Lemon* construct possessed half of the *Lemon* extracellular domain and the *Lemon* transmembrane and kinase domains. The HTK7 and HTK54 constructs contained their respective transmembrane and kinase domains but lacked all but the juxtamembrane portion of the extracellular domain. Yeast containing the various constructs were induced with galactose for 14 h. Identical amounts of total protein were fractionated by SDS–PAGE, transferred to a PVDF filter, and probed with an anti-myc antibody. The level of protein expression from the construct containing all *Lemon* sequences was dramatically reduced relative to the levels of protein from the HTK7 and HTK54 constructs (Fig. 4). To determine if the *Lemon* transmembrane sequence played a role in this reduction in protein level, we expressed two transmembrane sequence chimeras. In the first chimera, the transmembrane region (transmembrane sequence and 10 amino acids on each side) of *Lemon* was replaced with the equivalent region from HTK7. In the second chimera, the transmembrane region of HTK54 was replaced with the equivalent region from *Lemon*. To examine the stability of these chimeric proteins, we performed a glucose repression experiment. Transformed yeast strains were grown in 2% galactose for 14 h; 3% glucose was then added to repress the GAL promoter and block further RTK synthesis. Samples were taken at various times following glucose repression and analyzed by immunoblotting. Figure 4 shows that proteins containing the *Lemon* transmembrane sequence exhibit a reduction in protein stability relative to controls. Replacing the *Lemon* transmembrane region with the HTK7 transmembrane region in this construct resulted in an increase in the protein level (Fig. 4C). Taken together, these results indicate that the presence of the *Lemon* transmembrane sequence decreases protein stability.

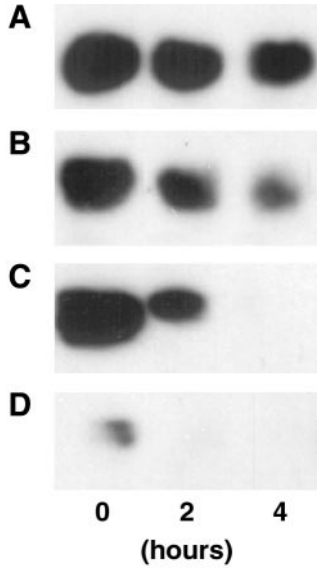


FIG. 4. The *Lemon* transmembrane sequence contains a destabilizing element. Yeast strains containing myc-tagged *Lemon* transmembrane sequence chimeras were grown in medium containing 2% galactose for 14 h. 3% glucose was then added and samples were taken after 0, 2, and 4 h. Protein extracts from each sample were fractionated by SDS–PAGE, transferred to a filter, and probed with the 9E10 anti-myc monoclonal antibody. (A) HTK54; (B) HTK54 possessing the *Lemon* transmembrane sequence; (C) *Lemon* possessing the HTK7 transmembrane sequence; (D) *Lemon* with its own transmembrane sequence.

The Lemon Gene Is Dynamically Expressed in Interstitial Cells

The expression pattern of the *Lemon* gene was examined using Northern analysis and *in situ* hybridizations to whole *Hydra* polyps and to macerated cell preparations of testes and ovaries. *In situ* hybridizations to macerated cell preparations of body column or bud tissue could not be resolved due to a poor signal-to-background ratio. The *Lemon* expression pattern was found to be dynamic and complex, with *Lemon* RNA being detected in two classes of cells, tentacle endodermal cells (Fig. 5O) and interstitial cells. Northern analysis, which was performed using mRNA isolated from dissociated cell fractions separated by centrifugal elutriation (generously provided by Drs. Thomas Holstein and Bert Hobmeyer, University of Darmstadt), also supported this localization (data not shown). *Lemon* mRNA is present at low levels in interstitial cells in nonsexual, nonbudding polyps (Fig. 5A) and at higher levels in these cells during budding (Figs. 5B–5D), spermatogenesis, and oogenesis (Figs. 5E–5N).

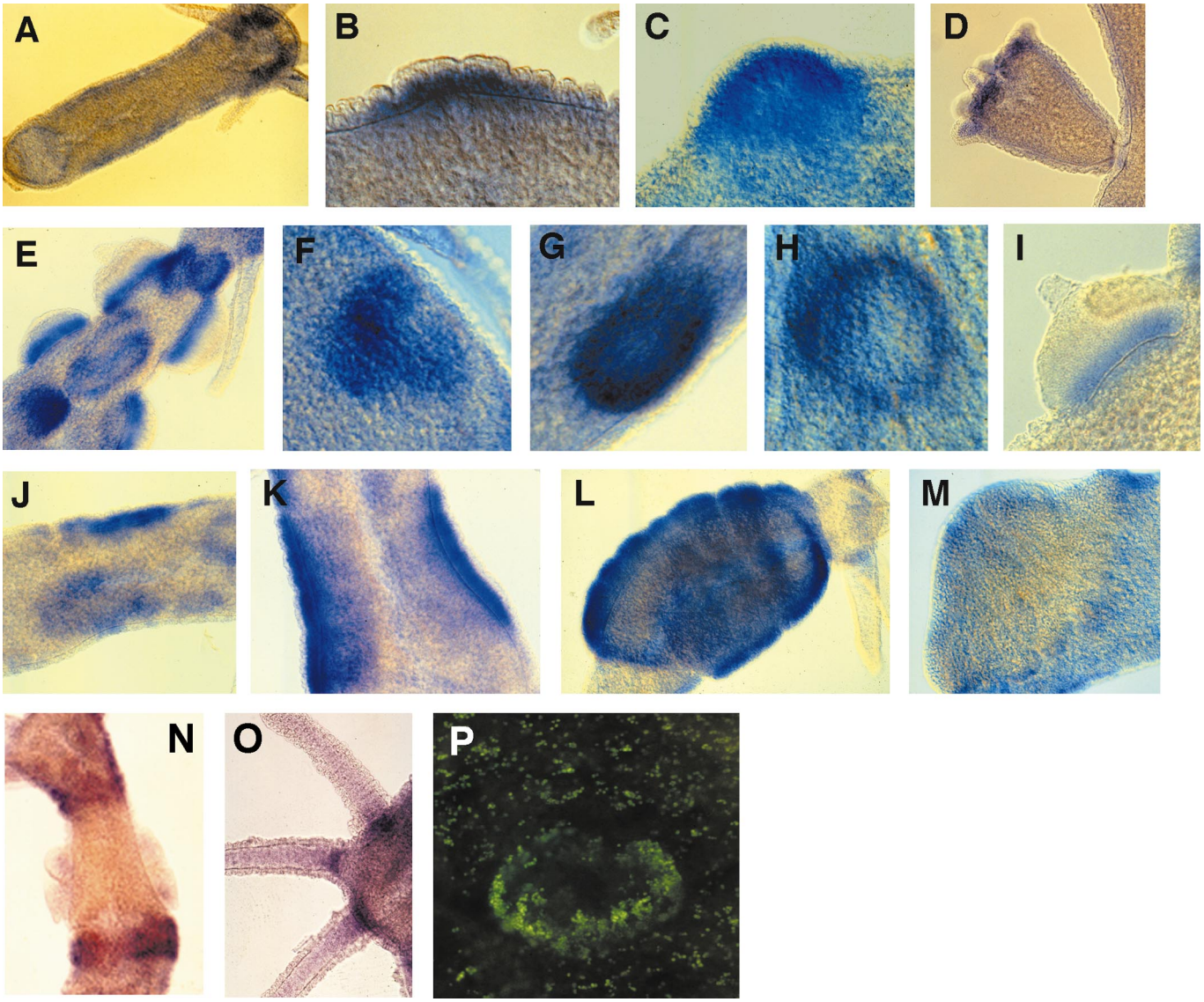
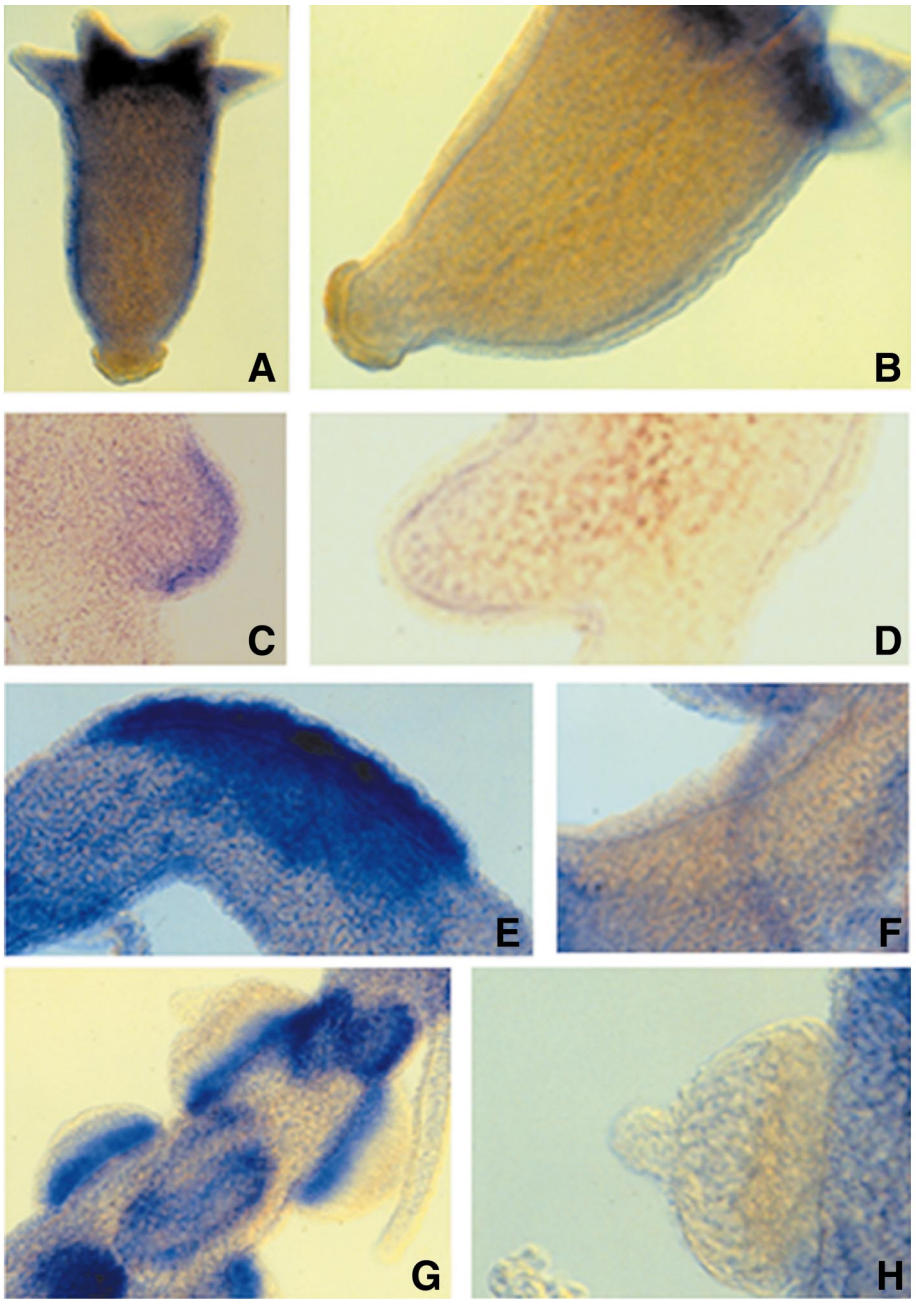


FIG. 5. Whole-mount *in situ* hybridization analysis of *Lemon* gene expression. (A) A nonsexual adult polyp. Close-up views of a stage 2 bud (B), a stage 3 bud (C), and a stage 7 bud (D). Bud stages are according to Otto and Campbell (1977). The body column of a male polyp with numerous testes is shown in (E). Close-up views of developing testes are shown in F–I. In (F), interstitial cells that have up-regulated *Lemon* expression have accumulated and mark the future site of the testis. *Lemon* expression then fades from the center of the accumulation (G and H) and becomes confined to the basal periphery of the mature testis (I). During oogenesis, *Lemon* expression is up-regulated in large patches of aggregating interstitial cells in the female body column (J). These patches of expression grow (K) until much of the body column is stained (L). As the nurse cells undergo apoptosis and become phagocytosed by the oocyte, *Lemon* expression dissipates (M). After the oocyte is externalized, *Lemon* expression is absent from the body column where the egg field existed (N). (O) A close-up view of *Lemon* expression in tentacles. BrdU-pulse labeling of a mature testis shows a ring of cells in S phase (P) that matches the *Lemon* expression pattern.

In *Hydra*, interstitial cells are located primarily between ectodermal epithelial cells; hence, it can be difficult to distinguish *in situ* hybridization to ectodermal epithelial cells from hybridization to interstitial cells. To distinguish between these two possibilities, *in situ* hybridizations to normal animals can be compared to those done with animals that have been treated with hydroxyurea (HU) to selectively eliminate the interstitial cells (Bode *et al.*, 1976). Treating polyps for 3 days is usually sufficient to reduce the interstitial cell population to about 5% of its normal value. In order to eliminate differentiating interstitial cells that do not cycle or cycle slowly, one must remove the proliferating



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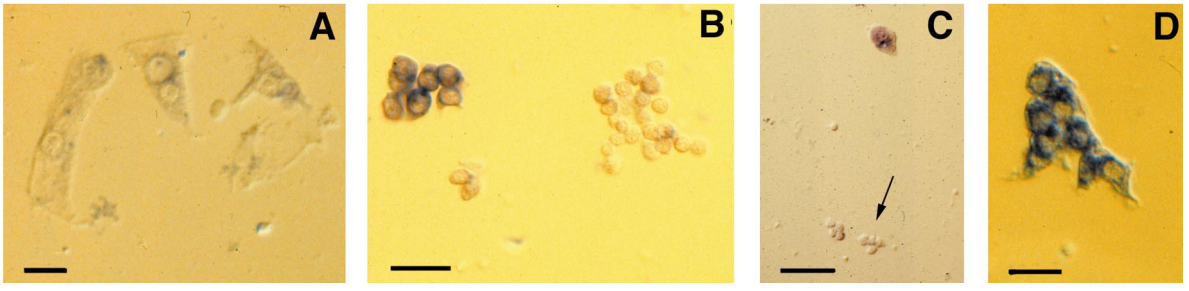

7

stem cells first, using HU, and then wait several days to weeks for the differentiation products to be lost by displacement from the extremities of the polyp. Since there are no stem cells remaining, the entire interstitial cell population will be lost.

Nonsexual budding, nonbudding sexual male, and sexual female polyps were treated with HU for 1 or 3 days, allowed to recover for 2 days, and then analyzed for *Lemon* mRNA expression using whole-mount *in situ* hybridization (Fig. 6). The effectiveness of the HU treatment was confirmed by counting cells from macerated polyps to determine the interstitial cell to epithelial cell ratio. As interstitial cells were progressively removed by epithelial cell phagocytosis in HU-treated animals, *Lemon* mRNA expression was lost. In most cases, 3 days of HU treatment eliminated all but the expression within tentacle endodermal cells (compare Figs. 6A and 6B, 6C and 6D). The interstitial cells with elevated levels of *Lemon* expression in sexual animals are more sensitive to HU than other interstitial cells. For example, in animals treated for 1 day with HU, expression in testes disappeared while expression was still seen in body column interstitial cells (compare Figs. 6G and 6H). A similar phenomenon was seen in sexual females treated for 3 days with HU (compare Figs. 6E and 6F). Additionally, in the region of the body column of untreated animals where the interstitial cell density is low or where interstitial cells are not present, *Lemon* expression is reduced or absent. For example, *Lemon* expression is absent in the region of the body column which is depleted of interstitial cells by the process of oogenesis (Fig. 5N). Taken together, these results indicate that *Lemon* is predominantly expressed in interstitial cells.

In sexual male polyps, *Lemon* transcriptional up-regulation occurs in patches of sperm-restricted interstitial cells located in the body column. These interstitial cells, which accumulate concurrently with *Lemon* transcriptional up-regulation, mark the initial formation of the testis (Fig. 5F). During spermatogenesis, the *Lemon* expression pattern changes as the transcript disappears from the center of the aggregating mass of interstitial cells and becomes gradually restricted to a thin ring around the basal periphery of the mature testis (Fig. 5G–I). Previous descriptions of the architecture within the testis (Brien and Reniers-Decoen, 1950) were insufficient to explain this *Lemon* expression pattern. To resolve this problem, sexual male polyps were pulse labeled with BrdU to identify cycling cells and stained with toluidine blue to visualize interstitial cell types within the testis. We found that as the testis develops, proliferating cells gradually become restricted to the periphery of the structure. In a mature testis, the spermatogonia, which are proliferating cells, are located adjacent to the surrounding ectodermal cells in a basal ring (Fig. 5P). This pattern is identical to the *Lemon* expression pattern, supporting the idea that expression of *Lemon* mRNA is correlated with proliferation in the spermatogenic lineage.

In order to identify specific cell types that express *Lemon* during spermatogenesis, *in situ* hybridizations were performed on cells from dissected, macerated testes. Within the testis, *Lemon* RNA was detected in large interstitial cells (Fig. 7B) and a subset of small interstitial cells, but was undetectable in the epithelial cells (Fig. 7A) and the majority of small interstitial cells, spermatids, and sperm (Figs. 7B and 7C). This expression pattern is consistent with the whole-mount *in situ* hybridization data and with previous data that defined which cells in the spermatogenic lineage proliferate (Munck and David, 1985).

Whole-mount *in situ* hybridization indicates that *Lemon* transcriptional up-regulation occurs during oogenesis in patches of accumulating interstitial cells similar to those seen during spermatogenesis (Fig. 5J). However, during oogenesis these patches grow in size until most of the body column is intensely stained (Figs. 5K and 5L). During oogenesis, thousands of interstitial cells accumulate and proliferate (Honegger, 1981; Tardent, 1985). One cell located near the center of the accumulation is selected to be the oocyte while all others differentiate into nurse cells and are phagocytosed by the growing oocyte (Honegger, 1981).

In situ hybridizations done on sexual female polyps confirm that *Lemon* transcription is up-regulated in the accumulating interstitial cells (Fig. 7D). Because the single oocyte cell is morphologically indistinguishable from the thousands of nurse cells during this early stage of female gametogenesis, we were unable to determine if the level of *Lemon* RNA drops in the differentiating oocyte. The *Lemon* RNA level in nurse cells does, however, drop shortly after nurse cells are transformed into apoptotic bodies and phagocytosed by the oocyte (Fig. 5M). After the oocyte is externalized, there is a temporary void of interstitial cells in the region of the body column where the oocyte was produced. *Lemon*-expressing cells are absent from this region (Fig.

FIG. 6. Effect of hydroxyurea (HU) treatment on *Lemon*-expressing cells. (A) Untreated nonsexual polyp. (B) Nonsexual polyp treated with HU for 3 days. (C) Stage 3 bud on an untreated polyp. (D) Stage 5 bud on a polyp treated with HU for 3 days. (E) Untreated sexual female polyp. (F) Sexual female polyp treated with HU for 3 days. (G) Untreated sexual male polyp. (H) Sexual male polyp treated with HU for 1 day.

FIG. 7. *In situ* hybridization analysis of *Lemon* expression in cells from dissected and macerated testes and regions of the body column containing developing oocytes. (A) Epithelial cells from the body column of a sexual male polyp. Larger spermatogonia express *Lemon* (cells on the left in B), but a subset of smaller spermatogonia (cells on the right in B) as well as smaller differentiating sperm precursors (C, arrow) do not. During oogenesis, *Lemon* is up-regulated in aggregating interstitial cells (D), all but one of which will differentiate into nurse cells (see text for explanation). Bars represent 20 μm.

5N), which adds support to our results with HU-treated animals demonstrating that *Lemon* expression in the body column is restricted to interstitial cells.

DISCUSSION

Phylogenetic analysis strongly supports the hypothesis that *Lemon* is orthologous to *Drosophila* Dtrk, chicken Klg, and human CCK-4 and thus places the origin of this family of unusual RTKs at a point deep in the metazoan radiation. The members of the family share three distinguishing features. First, their extracellular domains contain only immunoglobulin-like repeats; second, their transmembrane domains are highly conserved, a feature which is so far unique among RTKs; and third, their kinase domains have alterations in the highly conserved DFG motif. The aspartate of this motif, which is located within the active site, functions in the chelation of Mg^{2+} during phosphotransfer and has been shown to be essential for kinase activity (Moran *et al.*, 1988; Taylor *et al.*, 1993; van der Geer *et al.*, 1994). Dimerization of RTKs brings one catalytic domain in close proximity to another, resulting in *trans*-phosphorylation. Residues important for ATP binding hold the ATP–Mg^{2+} complex near the active site while the aspartate of the DFG motif orients the complex for phosphotransfer (Taylor *et al.*, 1993). Another aspartate residue within the active site serves as the catalytic base. Most other conserved residues are believed to be involved in substrate recognition or to serve structural roles (reviewed in van der Geer *et al.*, 1994). Since *Lemon,* Klg, CCK-4, and Dtrk have alterations in a motif that is known to be essential for kinase activity, we hypothesized that the signal transduction mechanism of these orthologues is atypical. In support of this idea, kinase activity could not be detected in *Lemon,* CCK-4, and Klg (Chou and Hayman, 1991; Mossie *et al.*, 1995). In contrast, an immunoprecipitate of *Drosophila* Dtrk was capable of phosphorylation (Pulido *et al.*, 1992); however, the possibility that this activity was due to a coprecipitating kinase was not ruled out. Although one could argue that the substrate specificities of *Lemon,* Klg, and CCK-4 are peculiar and therefore not easily assayed, our present knowledge of RTKs argues against this hypothesis since all known catalytically active RTKs recognize themselves as substrates.

We speculate that *Lemon* is a component of an unusual RTK signal transduction mechanism involving the formation of a complex which is regulated, in part, by transmembrane domain interactions. This hypothetical complex would consist of a kinase-inactive receptor and an unidentified RTK possessing protein-tyrosine kinase activity. A similar mechanism has been demonstrated for the vertebrate RTK ErbB3, which lacks or has a very low level of protein-tyrosine kinase activity (Guy *et al.*, 1994). ErbB3 forms heterodimers with other members of the epidermal growth factor receptor family like EGFR and ErbB2 (reviewed in Riese and Stern, 1998). These heterodimers have different signaling capabilities relative to EGFR and ErbB2 homodimers (Alimandi *et al.*, 1995; Fedi *et al.*, 1994).

This heterodimerization hypothesis is supported by several observations and results. *Lemon,* Klg, and CCK-4 are orthologues that do not possess catalytic activity, suggesting that this activity was lost before cnidarians split from the common ancestor. Despite this loss of enzymatic activity, their kinase domains have remained relatively conserved, conservation that may be necessary to facilitate an interaction with another protein-tyrosine kinase domain. We speculate that there has not been selection for *Lemon* catalytic activity during cnidarian evolution and hence, the absence of kinase activity is no longer due only to a single change in the amino acid sequence. In keeping with this notion, conversion of the FLS motif to its ancestral DFG sequence was not sufficient to restore kinase activity.

The highly conserved transmembrane sequence of *Lemon* and its orthologues suggests that this sequence plays an important role in the function of these proteins. We have showed that the *Lemon* transmembrane sequence contains a destabilizing determinant when expressed in yeast. Transmembrane sequences have been shown to play roles in the assembly of membrane protein complexes (Blum *et al.*, 1993; Bonifacino *et al.*, 1990a,b; Cosson and Bonifacino, 1992; Harrison *et al.*, 1995; Hennecke and Cosson, 1993), and in at least some cases failure of the transmembrane sequence to interact with its binding partner leads to rapid degradation of the protein in the endoplasmic reticulum (Bonifacino *et al.*, 1990a,b). It is possible that the effect of the *Lemon* transmembrane sequence on protein stability is achieved through a similar mechanism.

Interestingly, the amino acid sequence motifs present in the transmembrane domains of *Lemon* and its orthologues are very similar to the motifs present in the transmembrane domain of glycophorin A, a prominent glycoprotein in human erythrocyte membranes which forms noncovalent homodimers mediated by specific sequences within its transmembrane domain (Engelman *et al.*, 1995; Fleming *et al.*, 1997; Lemmon *et al.*, 1992; MacKenzie *et al.*, 1997). Another *Hydra* RTK, HTK54 (Entrez No. AAA65223), possesses related motifs in its transmembrane domain, raising the possibility that this protein forms a complex with *Lemon* which is mediated by transmembrane sequence interactions. The expression pattern of HTK54, which is a *Hydra* relative of the vertebrate PDGF receptor family (Fig. 2), overlaps the expression pattern of *Lemon* (data not shown). Preliminary results suggest that the coexpression of *Lemon* and HTK54 in yeast results in an increase in the steady-state level of the *Lemon* protein (data not shown). We are currently conducting an investigation into the nature of this putative interaction.

Although the role of *Lemon* in gametogenesis is unknown, its expression pattern in interstitial cells suggests at least one possible function. In *Hydra,* gametogenesis initiates as sperm- or egg-restricted interstitial cells accumulate in localized regions of the body column. Prior to this accumulation, these cells proliferate slowly, going through

S-phase every 3 to 4 days (Holstein and David, 1990; Littlefield, 1991; Littlefield *et al.*, 1991). However, sperm-restricted interstitial cells enter S phase every 16 to 24 h during spermatogenesis (Munck and David, 1985). Egg-restricted interstitial cells proliferate at similar rates during oogenesis based on their rapid incorporation of BrdU and elimination with HU, but accurate labeling analyses are difficult because there are no antibodies that distinguish egg-restricted interstitial cells from other interstitial cell types. *Lemon* transcriptional up-regulation during spermatogenesis and oogenesis occurs early, when sperm- or egg-restricted interstitial cells initially accumulate. Whole-mount BrdU labeling indicates that these initial accumulations consist of rapidly proliferating interstitial cells (Miller, unpublished observations). Thus, *Lemon* transcriptional up-regulation occurs concurrent with an increase in the proliferation rates of sperm- or egg-restricted interstitial cells early in gametogenesis. In addition, interstitial cells up-regulating *Lemon* transcription during budding have altered cell cycle parameters relative to interstitial cells down-regulating *Lemon* transcription in the budding zone of the parent polyp (data not shown). These correlations suggest that *Lemon* may be involved in modulating the cell cycle of interstitial cells.

CCK-4, the human orthologue of *Lemon*, was originally identified as a gene expressed in primary colon carcinoma tissue (Mossie *et al.*, 1995). CCK-4 is not expressed in normal adult colon, and its expression was found to be elevated in 9 of 15 colon carcinoma cell lines (Mossie *et al.*, 1995). Interestingly, high levels of expression of the murine orthologue of CCK-4 are seen in fetal mouse colon (Mossie *et al.*, 1995). CCK-4 (in this case termed PTK7) is also expressed in normal melanocytes, but expression was either decreased or absent in advanced melanomas (Easty *et al.*, 1997). Why expression of CCK-4 is elevated in one type of tumor and suppressed in another type is unclear. These data indicate, however, that it will be of interest to examine in more detail the members of this unusual family of RTKs.

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