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Short sequence-paper

Zebrafish cyclin D1 is differentially expressed during early embryogenesis [1]

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Abstract

We have isolated and determined the nucleotide sequence of a cDNA containing the complete coding region of cyclin D1 from embryonic zebrafish cDNA library. The cyclin D1 gene is a single copy gene within the zebrafish genome, which undergoes an alternative polyadenylation process. The initial expression of cyclin D1 transcript occurs at the presumed onset of G1 phase in the developing zebrafish embryo.

Keywords: Cyclin; Embryogenesis; Growth control; (Zebrafish)

The G1 cyclins and their dependent kinases (cdks), which have been suggested to function at the G1/S phase transition, have also been shown to play an important role in cell differentiation and transformation [1]. Among the G1 cyclins, the D-cyclins (D1, D2 and D3) constitute a gene family whose members associate with several cdks, including cdk2, cdk4 and cdk6 [2,3]. The D-cyclins are induced by growth factors [4,5] and are differentially expressed in different mammalian cell lines and tissues [5,4,6–8]. In order to determine if their expression patterns could be correlated to the differentiation and cell cycle status of specific cells and tissues during early embryonic development, we studied cyclin D1 expression in zebrafish (*Danio rerio*). The zebrafish embryo is an especially attractive organism for such studies, since it enables analysis of molecular developmental processes in '4-dimensions' (3-dimensional analysis in time), due to its transparency and rapid extracorporal development [9].

For isolating the zebrafish cyclin D1, we screened a gastrula stage zebrafish cDNA library with a 0.6 kb *NcoI* fragment of the human cyclin D1 cDNA [10] as a probe. Under low stringency conditions [11] approximately 14 out of the 400000 plaques hybridized with the probe. Sequence analyses revealed that 13 of them encode a predicted aa sequence which is very similar to the human cyclin D1 polypeptide. Complete DNA sequence of two clones (pDrcycD1-102 and pDrcycD1-132) which included the predicted initiator ATG, revealed that they are independent clones representing the same gene (Fig. 1). The clones are completely identical in the overlapping regions, but have different 5′- and 3′-ends (Fig. 1B). A consensus sequence for polyadenylation (AAUAAA) followed by a GU rich sequence [12] is located 300 bp downstream from the stop codon in both cDNAs. The 3′-non-coding sequence of pDrcycD1-102 is shorter than the corresponding area in pDrcycD1-132 and includes a stretch of poly-A (34 residues) immediately downstream from a polyadenylation signal. In pDrcycD1-132, there is no additional consensus sequence for polyadenylation nor a long stretch of poly-A and therefore, it may represent a 3′-truncated cDNA. The heterogeneity in the 3′-untranslated sequences among those clones suggests alternative polyadenylation of the transcript in zebrafish, as previously suggested for the human cyclin D1 [6] and the murine cyclin D2 [8] mRNAs. At the 5′-end, an inframe stop codon at position 153 upstream from the predicted initiator ATG, was found in clone pDrcycD1-132. Primer extension analyses showed that the 5′-ends are not longer then the 5′-ends of the pDrcycD1-132 cDNA (not shown). Therefore, there is no heterogeneity in

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[1] The nucleotide sequence data reported in this paper will appear in the EMBL, GenBank and DDJB Nucleotide Sequence Databases under the accession number X87581.

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the zebrafish cyclin D1 transcript length at the 5'-end, and the shorter clone pDrcycD1-102 was probably created during library construction which involved *XhoI* digest.

The cloned cDNAs encode a predicted 291-residue polypeptide with a calculated molar mass of 33 067 dalton

A

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-197  tctcgctctctcacaggctcgctgctgctgctttgatcttcccttaacaacagtcacgtc  -138
-137  ataggggagttttgtcaagcggagagctgcagcgagattttagagggttcagcagaggca  -78
 -77  gcaaaaagcatccacgggaccagtgtccgactcgagctccagctttcttacttttctcac  -18
 -17  tgctaaacactgaagatATGGAGCACCAGTTGTTTTGCTGCGAAGTGGATACCATAAGAA   42
                    M  E  H  Q  L  F  C  C  E  V  D  T  I  R  R
  43  GAGCTTACCAAGACAGCAACCTGTTGAATGACCGAGTTTTACAGACAATGCTTAAAGCTG  102
        A  Y  Q  D  S  N  L  L  N  D  R  V  L  Q  T  M  L  K  A  E
 103  AGGAAAACTATCTCCCATCACCAAATTATTTCAAGTGTGTTCAGAAAGAAATTGTGCCTA  162
        E  N  Y  L  P  S  P  N  Y  F  K  C  V  Q  K  E  I  V  P  K
 163  AAATGAGGAAAATCGTCGCGACGTGGATGCTCGAGGTCTGTGAAGAGCAGAAATGTGAAG  222
        M  R  K  I  V  A  T  W  M  L  E  V  C  E  E  Q  K  C  E  E
 223  AGGAAGTTTTTCCTTTGGCTATGAACTACCTGGACAGGTTTTTATCTGTGGAGCCCACCA  282
        E  V  F  F  L  A  M  N  Y  L  D  R  F  L  S  V  E  P  T  K
 283  AAAAAACCAGGTTGCAGCTTTTAGGAGCAACTTGTATGTTTCTGGCTTCAAAAATGAAAG  342
        K  T  R  L  Q  L  L  G  A  T  C  M  F  L  A  S  K  M  K  E
 343  AGACTGTGCCACTTACAGCAGAGAAGTTGTGCATATACACGGACAACTCTGTCCGTCCCG  402
        T  V  P  L  T  A  E  K  L  C  I  Y  T  D  N  S  V  R  P  G
 403  GCGAATTATTGCAAATGGAACTGCTGGCGCTAAATAAACTGAAGTGGGATCTGGCCTCAG  462
        E  L  L  Q  M  E  L  L  A  L  N  K  L  K  W  D  L  A  S  V
 463  TGACACCACATGATTTCATTGAACACTTCCTTGCCAAACTGCCTATACATCAGAGCTCCA  522
        T  P  H  D  F  I  E  H  F  L  A  K  L  P  I  H  Q  S  S  K
 523  AGCAGATACTGCGCAAACACGCCCAGACCTTTGTGGCCCTCTGTGCGACAGACGTCAACT  582
        Q  I  L  R  K  H  A  Q  T  F  V  A  L  C  A  T  D  V  N  F
 583  TCATCGCAAGCCCTCCCTCCATGATTGCAGCAGGCAGTGTTGCTGCAGCGGTACAAGGAC  642
        I  A  S  P  P  S  M  I  A  A  G  S  V  A  A  A  V  Q  G  L
 643  TGTACCTGAAAAGCACCGACAGTTGCCTCTCATCCCAGAACCTCACCAACTTCCTCTCGC  702
        Y  L  K  S  T  D  S  C  L  S  S  Q  N  L  T  N  F  L  S  Q
 703  AAGTCATCAGAAGTGACCCTGACTGTCTGCGATCGTGTCAGGAACAGATCGAGTCCCTGC  762
        V  I  R  S  D  P  D  C  L  R  S  C  Q  E  Q  I  E  S  L  L
 763  TGGAATCGAGTCTCAGACAAGCTCAGCAGCACATCTCCACAGAGACCAAAAGGGTCGAGG  822
        E  S  S  L  R  Q  A  Q  Q  H  I  S  T  E  T  K  R  V  E  E
 823  AGGACGTGGACCCTCTTGCACTCCCACAGACGTCAGAGACATTAACATTTGAagggatc   882
        D  V  D  L  S  C  T  P  T  D  V  R  D  I  N  I  *
 883  gctattcatctcgtgtgtgatgaatctcttcgtctttgtttctgatgggtatgggctttg  942
 943  gccaaaaggcgtcatgggacaagagggaaaacgggctcctacctgcaccccaccatctga 1002
1003  ccaatacctcgaaaacttgcccactcagcacgcccaaactgttgactgctgagcggcatc 1062
1063  ccctgtggctttgatgaccgagttatcgctggccaggaccaccggcgaatgacagggaat 1122
1123  gaatggaaattttaattttctcccttggccacccctttctatgtcctaagtcataataaa 1182
1183  atggtataggctatctaggattttacaaattgtataaatgatacaacagtagttgtgact 1242
1243  tgccttgacttgtcgctaaaaagacaaccatgggtatttttttatttcaagtgtgtcaaac 1302
1303  tatttttgaaagagaagagacattattttgtatgttcgatttctttctctcatagacagt 1362
1363  accttgttacacttgctacaagtgctccctgctttttctgcagtctagccgtgtaaatat 1422
1423  acccccgcttgctatgcgtttagtcactttattataattcctagacagattccctaaca 1482
1483  agattctcttctcttccaaaatggtttatgtaaccttagtatatttttttacgcatgtatt 1542
1543  tttgttatgatctagaaggctgtggattgcctgggggctgaggttctaactcgaggccaa 1602
1603  accgaaccatttctttgaattgttgccttcactgccattaatgtataatgtcatcactgc 1662
1663  catgacaaaataagcttacgttgtacctgctcccccagcagtgcagtccacagtccgctt 1722
1723  tgtgtttagattcatgtattatctaaaaaccattccatttaaaaaagaaaaaaagcactt 1782
1783  tcagtccgtcgtttaaggctgacttttaga 1812
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B

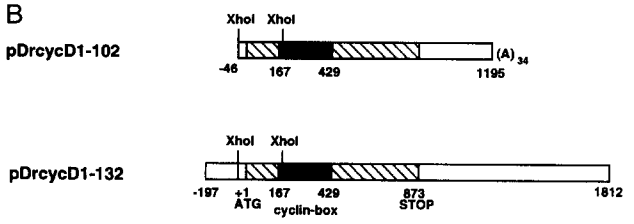

Fig. 1. Sequence of the zebrafish cyclin D1 cDNA and molecular structure of the alternatively polyadenylated zebrafish cyclin D1 cDNAs. (A) Complete nucleotide sequence of the zebrafish cyclin D1 cDNA pDrcycD1-132. The presumed cyclin box and polyadenylation signal are shown in bold print, PEST-rich sequence and LXCXE motif are underlined. (B) Molecular structure and alternative polyadenylation of the zebrafish cyclin D1 cDNAs. Open boxes represent non-coding sequences, hatched boxes represent coding sequences and black boxes represent the cyclin box.

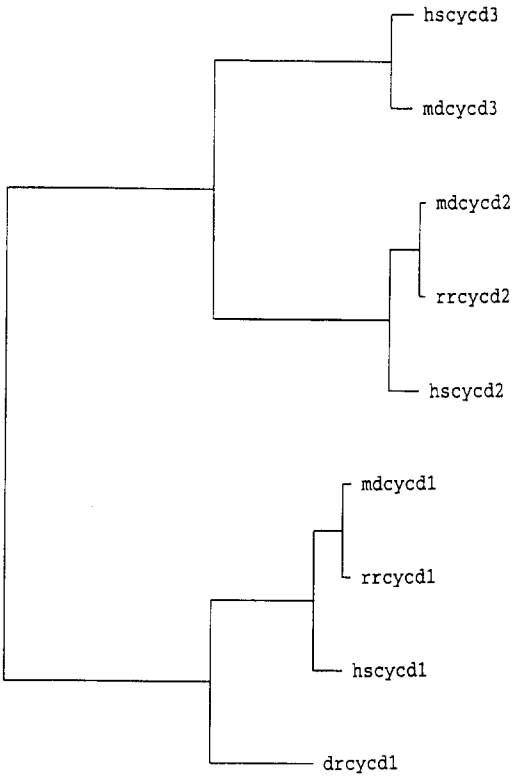

Fig. 2. Dendogram on the basis of multiple sequence alignment of D-type cyclins: drcycdl-zebrafish cyclin D1; hscycdl, 2- or 3-human cyclin D1 [6], 2 [21] or 3 [22], respectively; mdcycd1, 2- or 3-mouse cyclin D1, 2 or 3 [4], respectively; rrcycd1 or 2-rat cyclin D1 (accession #D14014) or 2 [23]. The tree and dendogram were calculated by the PILEUP and Distances programs in the GCG package, from deduced amino acid sequences. Branch length is relative to the number of amino acid substitutions. Neither rigorous calculation of evolutionary distances nor phylogenetic relationship can be inferred with confidence from this tree.

which carries the hallmarks of G1 cyclins [3]; namely, a conserved 'cyclin box' in the center region of the polypeptide, and a PEST-rich sequence at the C-terminus (Fig. 1). The predicted amino acid sequence of the cDNA shows a higher degree of aa identity to the human, mouse and rat cyclin D1, than to cyclin D2 or D3 (Fig. 2) or to other cyclins (not shown). It is 77% identical to the human and mouse cyclin D1, and only 63% and 55% identical to the human cyclin D2 and human cyclin D3, respectively. The predicted cyclin-box of 83 aa, which is located at aa 46-129, is 88% and 92% identical to the human and mouse cyclin D1, respectively. The regions which flank the cyclin box are approximately 70% identical to the human and

mouse cyclin D1 polypeptides. A conserved sequence, LXCXE, which is shared by DNA viral oncoproteins that bind Rb and p107 and the human D-type cyclins [13,14], is present at aa position 5-9, near the N-terminus (Fig. 1). The zebrafish cyclin D1 cDNA is a single copy gene within the zebrafish genome, as it hybridized to a single band in Southern blot analysis of zebrafish genomic DNA (Fig. 3A).

We have studied the expression pattern of cyclin D1 RNA during zebrafish development by Northern analysis. Total RNA (extracted in 3 M LiCl, 6 M Urea, 0.1 g/100 ml SDS and 10 mM NaAc; washed in 4 M LiCl, 8 M Urea and Phenol/Chloroform extracted) from various developmental stages which was probed with a zebrafish cyclin D1 fragment, revealed two mRNA species of approximately 5 and 4 kb (Fig. 3B). These transcripts are longer then the isolated cDNA clones. One possible explanation for the lack of a signal at the expected size corresponding to the full length 1.2 kb pDrcycD1-102 clone is low abundance of this mRNA species during this phase of development. As pDrcycD1-132 is a 3′-truncated cDNA, it may represent part of the 4 and 5 kb RNA transcripts which were detected here. Since there is probably no heterogeneity in the 5′-non-coding sequences of cyclin D1 mRNA, the 4 kb and 5 kb size heterogeneity is probably determined by alternative processing of the 3′-untranslated region, which is not represented in the clones we have isolated.

Both zebrafish cyclin D1 RNA species are embryonic transcripts, first detected at the beginning of epiboly. The 4 kb mRNA stays relatively constant during epiboly, while the 5 kb RNA levels gradually increase and peak at 100% epiboly (Fig. 3B). Both transcripts decline during somitogenesis (Fig. 3B), reduced below detection levels at 48 h and are not detected at later stages of development (not shown). Methylene blue staining of the blot before hybridization, shows that equal amounts of RNA were loaded in each lane (Fig. 3C).

During early embryonic development precisely regulated switches in cell cycle length and pattern are well coordinated with morphogenesis and differentiation. In zebrafish the cleavage stage consists of 10 S/M cycles, followed by a gradual increase in cell cycle length [15]. At the 10th cleavage, which marks the beginning of the midblastula transition (MBT), lengthening of the cell cycle was shown to be controlled by the nucleo-cytoplasmic volume ratio [16,17]. The molecular basis for the loss of cell cycle synchrony at MBT [17], and the subsequent elongations which occur in the cell cycle pattern (Zamir, E., Kam, Z. and Yarden, A., unpublished data) are not known. Here we report that cyclin D1 mRNA is below detection levels at the initial S/M cycles of the early zebrafish blastula. This observation is in contrast to the presence of other G1 cyclins, such as cyclin C and cyclin E, in the initial S/M cycles of *Drosophila* and zebrafish [18–20]. Cyclin D1 is shown here to be the first non-maternally supplied G1 cyclin, which is induced only at the onset of epiboly in the zebrafish embryo. This observation may give the first indication for the initial appearance of a G1 phase in the developing zebrafish embryo.

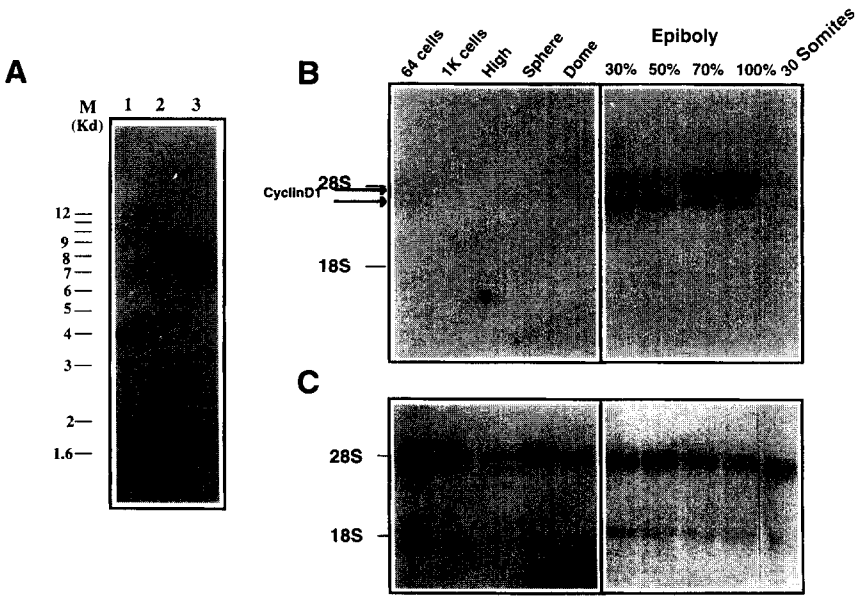

Fig. 3. Southern (A) and Northern (B)(C) blot analysis of the zebrafish cyclin D1. (A) Ten micrograms of total zebrafish genomic DNA were digested with *DraI* (lane 1), *EcoRI* (lane 2) or *PstI* (lane 3), blotted and probed with a hexamer labeled 1.2 kb (*AspI-BamHI*) fragment of pDrcycD1-102. Molecular weight markers were run alongside the gel and are marked on the left. (B) Five micrograms total RNA prepared from different developmental stages of zebrafish embryos (as marked) were resolved on an agarose gel, blotted to anylon membrane and hybridized to a hexamer labeled zebrafish cyclin D1 cDNA fragment (as in (A)). The 5 and 4 kb cyclin D1 mRNAs are marked with an arrow on the left. (C) The positions of the 28S and 18S rRNA which were visualized by methylene blue staining of the blot prior to hybridization.

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