DEVELOPMENTAL DYNAMICS 206:1–11 (1996)

Zebrafish Cyclin E Regulation During Early Embryogenesis

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ABSTRACT **Cyclin E cDNA, cloned from a zebrafish embryonic cDNA library, was used for analysis of cyclin E regulation during early embryogenesis. During the rapid cell cycles of the early cleavage stage, which lacks a G1 phase, the cyclin E mRNA, protein, and associated H1 kinase activity were found to be constitutive, in contrast to their reported cyclic behavior during the cycle of cultured mammalian cells. These results suggest an additional role for cyclin E during early embryogenesis, in addition to its established role during the G1/S transition in somatic cells. These results support previous identification of cyclin E in early cleaving *Drosophila* and *Xenopus* embryos, and provide for the first time the direct demonstration of constitutive cyclin E activity throughout the M/S cycles of the embryonic cleavage stage. Cyclin E mRNA was reduced during epiboly (approximately 6–8 hr postfertilization, HPF), concomitantly with a marked reduction in cell division rates. In contrast, the cyclin E protein and cyclin E-CDK complexes remained constant throughout the first 24 hr, implying that the cyclin E protein is regulated post translationally and is not immediately affected by the levels of the corresponding mRNA. However, the cyclin E-CDK complexes present in 26 somite embryos (22 HPF) did not exhibit histone H1 kinase activity. This discrepancy between high levels of cyclin E-CDK complexes and low enzymatic activity may be explained by the presence of putative cyclin E-CDK inhibitory mechanism. Here we show that multiple levels of regulation of the cyclin E mRNA, protein, and associated kinase activity are present during the first 24 hr of zebrafish embryonic development.** © 1996 Wiley-Liss, Inc.

Key words: Cyclin, Embryogenesis, Growth control, Zebrafish

INTRODUCTION

Controlled cell proliferation plays a central role in embryonic morphogenesis and differentiation. During early embryonic development, precisely regulated switches affect cell cycle lengths and patterns. Following fertilization, rapid and synchronous cleavages usually lead to the formation of a large population of blastomeres. In zebrafish (*Danio rerio*) the cleavage phase consists of 7 synchronous M/S cycles, after which the embryos show slight metasynchrony, with waves of mitosis emanating from the animal pole (Kimmel and Law, 1985; Kimmel et al., 1995). During this early blastula stage there is a gradual increase in the cell cycle length (Marrable, 1965). At the 10th cleavage, the beginning of the midblastula transition (MBT), lengthening of the cell cycle was shown to be controlled by the nucleo-cytoplasmic volume ratio (Kane and Kimmel, 1993; Kane et al., 1992), as previously found in other systems (Edgar et al., 1986; Newport and Kirschner, 1982). At this stage, three cell layers arise, each of them playing a separate role during epiboly and displaying distinct mitotic behavior (Kane et al., 1992). Two of these domains are extra-embryonic: the yolk syncytial layer nuclei were shown to divide most rapidly, and the enveloping epithelial layer most slowly. The third domain, which forms the entire embryo, consists of an inner mass of deep spherical cells which were reported to divide with similar rhythm through cycle 16 (Kane et al., 1992). At cycles 15 and 16 cell divisions and oriented intercalations lead to the formation of long strings of cells along the embryonic axis (Kimmel et al., 1994). While these phenomena were extensively described at the morphological level, the molecular basis for the loss of cell cycle synchrony at MBT and for the suggested cell cycle dependency during gastrulation and segmentation, is not understood.

A central control system which regulates cell cycle progression in eukaryotic cells, is driven by molecular complexes formed between different cyclins and cyclin-dependent-kinases (CDKs) (Hunt, 1991; Norbury and Nurse, 1992; Reed, 1992), which drive cells through major checkpoints in the cell cycle. In the yeasts *Schizosacchromyces pombe* and *Saccharomyces cerevisiae* only one CDK (CDK1), associated with different cyclins, controls the G1/S and G2/M transitions (Forsburg and Nurse, 1991). In higher eukaryotes, CDK1 associated with mitotic cyclins (cyclin A, B1, and B2) controls the G2/M transition, while multiple CDK-cyclin complexes control the G1/S transition (Pines, 1993; van den Heuvel and Harlow, 1993). The mitotic cyclins were initially identified in marine invertebrates and their accumulation and subsequent degradation were shown to drive cells in and out of mitosis (Evans et al.,

Received July 3, 1995; accepted November 13, 1995.
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1983; Glotzer et al., 1991; Murray and Kirschner, 1989). Putative G1 cyclins (cyclin C, D, and E) were isolated when human and *Drosophila* cDNA libraries were used to complement yeast G1 mutants (Lew et al., 1991; Lahue et al., 1991). Among the human G1 cyclins, only cyclin E was shown to fluctuate periodically through the cell cycle. The amounts of cyclin E mRNA, protein, and CDK2-associated protein kinase activity were maximal in late G1 in HeLa cells (Lew et al., 1991; Dulic et al., 1992). Functional analysis suggested that cyclin E is rate-limiting for G1 progression, since overexpression of cyclin E accelerated the G1/S transition in mammalian fibroblasts, while microinjection of anti-cyclin E antibodies inhibited this transition (Resnitzky et al., 1994; Ohtsubo et al., 1995). In addition, cyclin E was shown to be required for S phase entry and to specifically induce transcription of S phase genes (DNA polymerase α, PCNA, and two ribonucleotide reductase subunits) in *Drosophila* (Knoblich et al., 1994; Duronio and O'Farrell, 1994).

In addition to the fluctuations observed in the levels of the different cyclins along the cell cycle, the activity of the cyclin-CDK complexes is determined by post-translational control mechanisms (Solomon, 1993; Peter and Herskowitz, 1994; Sherr, 1994; Sherr and Roberts, 1995). Following the assembly of the cyclin E with CDK2, the complex is subjected to phosphorylation and dephosphorylation events which regulate its activity. Activation by CAK which phosphorylates a single threonine residue yields a functional holoenzyme (Fisher and Morgan, 1994; Makela et al., 1994), while inactivation by WEE1 kinase is followed by re-activation by CDC25A phosphatase (McGowan and Russell, 1995; Hoffmann et al., 1994). Another level of post-translational regulation involves the participation of specific inhibitory proteins which bind the cyclin-CDK complexes and sequester their activity (Peter and Herskowitz, 1994; Sherr and Roberts, 1995). The protein inhibitors p21, p27, and p57 were shown to bind directly to the cyclin E-CDK2 complex, inhibit its enzymatic activity, and induce cell cycle arrest (Sherr, 1994; Sherr and Roberts, 1995). Moreover, p21 was localized in postmitotic differentiated mouse embryos (Parker et al., 1995), in differentiating hematopoietic cells (Steinman et al., 1994) and in differentiating cultured myoblasts (Halevy et al., 1995), while p57 was also found in non-proliferating cells during mouse embryogenesis (Matsuoka et al., 1995) suggesting an important role for those inhibitors in the terminal differentiation associated growth arrest.

Our studies focus on the regulation of cell proliferation during early embryogenesis of the zebrafish embryo. The zebrafish embryo is a particularly attractive organism for such studies since it enables analysis of developmental phenomena in "4-dimensions" (3-dimensional analysis in time), due to its transparency and rapid extracorporal development (Kimmel, 1989). We have recently showed that the zebrafish homologue of cyclin D1 is the first G1 cyclin reported not to be supplied maternally and induced only at the onset of epiboly in the zebrafish embryo (Yarden et al., 1995). This report describes the isolation and characterization of the zebrafish homologue of cyclin E (*DrcycE*) cDNA, which is a highly conserved molecule that is subjected to multiple levels of regulation during embryogenesis.

RESULTS

Cloning of the Zebrafish Cyclin E cDNA

In order to isolate the zebrafish cyclin E cDNA, we screened a gastrula-stage zebrafish cDNA library with a previously cloned zebrafish cyclin E genomic fragment (Yarden et al., 1994; accession no. X83595). Three identical 2.1 Kb cDNAs were isolated and one of them was fully sequenced on both strands (accession no. X83594). The predicted amino acid (aa) sequence was found to be very similar to the human and the *Xenopus* cyclin E polypeptides (see below), and therefore we designated this clone the zebrafish cyclin E (*DrcycE*) cDNA (Fig. 1).

While no stop codons were found upstream of the most 5′ ATG, we assume that this ATG encodes the initiator methionine. This is based on the size of the predicted open reading frame and the N-terminal sequence which is similar to that of the human and the *Xenopus* cyclin E molecules (Fig. 1). This most upstream ATG initiator was found to be preceded by a purine at position −3 (not shown) according to the expected Kozak consensus sequence for translation initiation by eukaryotic ribosomes (Kozak, 1986). A consensus sequence for polyadenylation (AAUAAA) followed by a GU rich sequence (Proudfoot, 1991) was found at the 3′ end of the cDNA (not shown). Another zebrafish cyclin E cDNA which was isolated in a parallel screen of an adult zebrafish cDNA library was shorter (1.2 Kb) and had truncated 5′ and 3′ ends. However, within the overlap, the sequences of the adult and the embryonic zebrafish cyclin E cDNAs, were identical (data not shown).

The zebrafish cyclin E cDNA encodes a predicted 410 amino acid residue polypeptide with a calculated molar mass of 46,630 dalton, which carries the hallmarks of G1 cyclins; namely a conserved "cyclin box" at the center of the molecule, and a PEST-rich sequence at the C-terminus (Sherr, 1993). The predicted amino-acid sequence of the zebrafish cyclin E is highly similar to that of the human and the *Xenopus* cyclin E (Fig. 1) exhibiting 60 and 55% identity respectively (Fig. 1), and 42% identity with the *Drosophila* cyclin E homologue (not shown). It contains a stretch of 91 aa (aa 145–236) which is 85% identical to the cyclin-box of the human cyclin E, including 5 highly conserved amino-acids found within all cyclin boxes (Hunt, 1991). The cyclin-box of the zebrafish cyclin E is 80 and 72% identical to the corresponding regions in *Xenopus* and *Drosophila,* respectively. Lower homology was found in other regions of the molecule which flank the cyclin box. The C-terminus, down-stream to the cyclin box (aa 236–410), is 60 and 50% identical to the human and

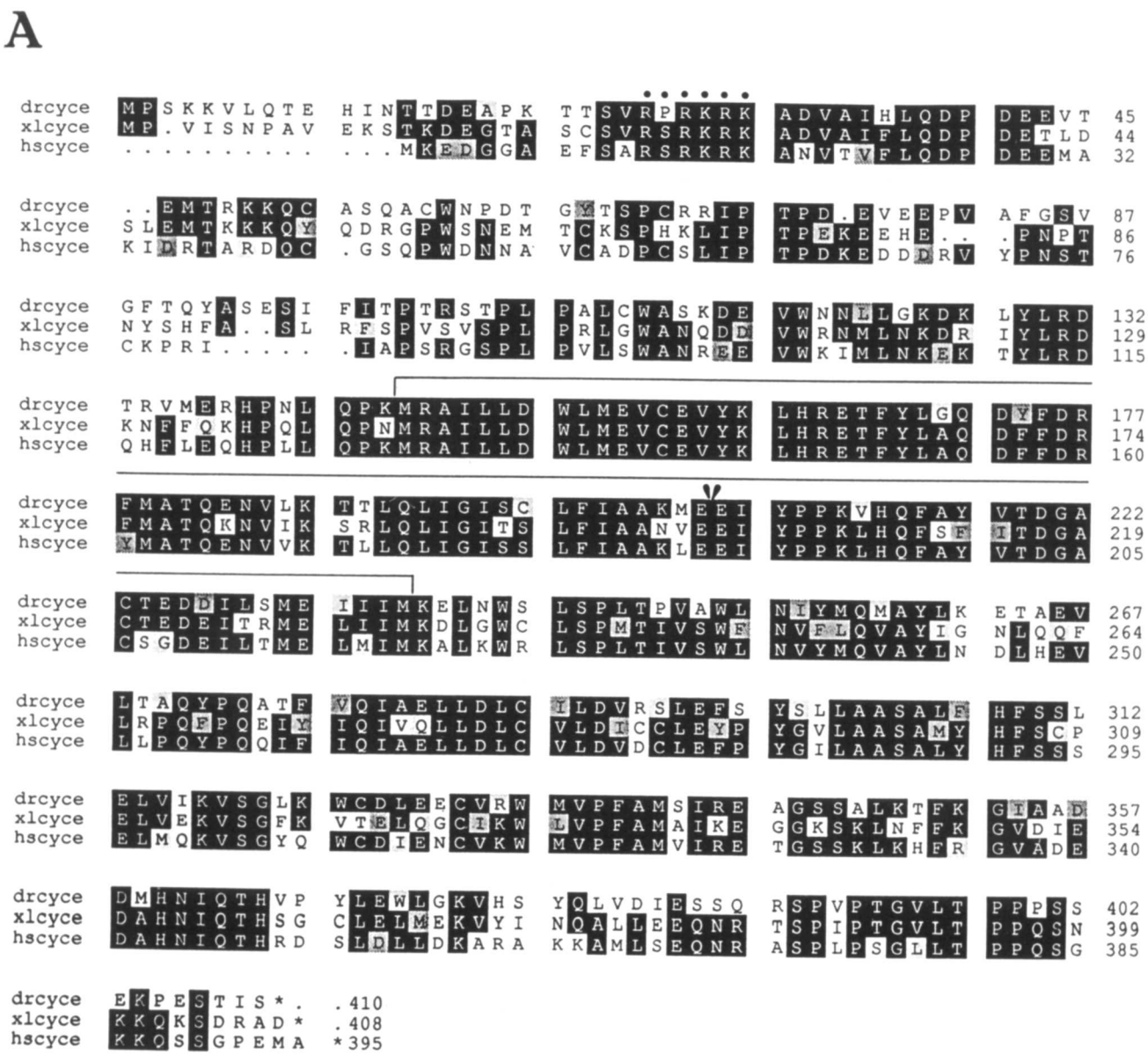

Fig. 1. **A:** Comparison between the zebrafish, *Xenopus,* and human cyclin E polypeptides. Alignment of the zebrafish (drcyce), *Xenopus* (xlcyce), and human (hscyce) predicted amino acid sequences. The *Xenopus* cyclin E is from the genebank accession no. L23857 (Rempel et al., 1995). The human cyclin E is from Lew et al. (1991). Black and gray boxes indicate identical and conservative amino acids between the zebrafish and the other two cyclin E polypeptides, respectively. The predicted nuclear localization signal is marked with bold dots and the cyclin box is underlined. Within the cyclin box the splicing point, deduced from the previously reported genomic fragment of the zebrafish cyclin E (Yarden et al., 1994), is marked with an arrowhead. **B:** Summary of the structure of the zebrafish cyclin E gene. Open boxes represent noncoding sequences, hatched boxes represent coding sequences, and black boxes represent the cyclin box.

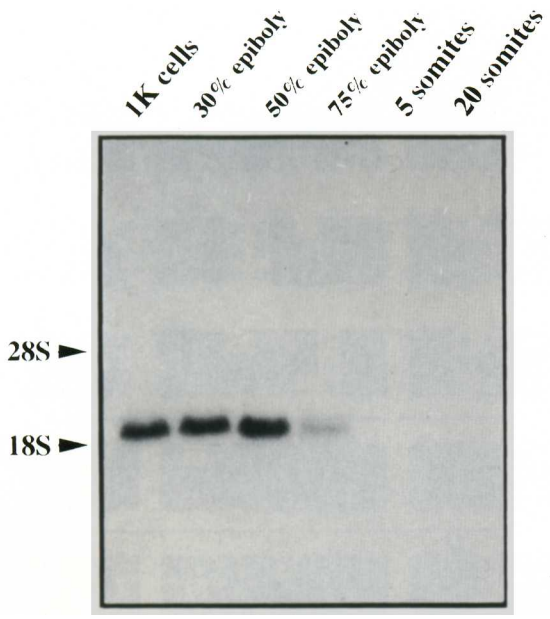

Fig. 2. Pattern of expression of the zebrafish cyclin E mRNA during zebrafish embryonic development. Northern blot of 10 μg total RNA, prepared from different developmental stages of zebrafish embryos and hybridized to the zebrafish cyclin E cDNA probe. The staging was done at 28°C according to Westerfield (1994) as follows: 1K cells (3 HPF), 30% epiboly (4.6 HPF), 50% epiboly (5.6 HPF), 75% epiboly (8 HPF), 5 somites (11.6 HPF), and 20 somites (19 HPF).

the *Xenopus* cyclin E, respectively. The N-terminus (aa 1–144) shows lower homology, with 43 and 45% identity to the human and the *Xenopus* proteins, respectively. At the N-terminus (aa 25–30) a potential nuclear localization signal (Garcia-Bustos et al., 1991) RPRKRK is located in a conserved box present in the zebrafish, human, and *Xenopus* molecules (Fig. 1).

Expression of Cyclin E mRNA in Zebrafish Embryos

We have studied the expression pattern of cyclin E mRNA during zebrafish embryonic development by Northern analysis. A single transcript of approximately 2.1 Kb was detected in total RNA which was prepared from various developmental stages and probed with a fragment corresponding to the zebrafish cyclin E cyclin box (Fig. 2). The size of this transcript is similar to that of the cDNA.

We have specifically analyzed RNA samples from the first 24 hr of zebrafish embryonic development (staged according to Westerfield, 1994). The developmental screening revealed constant levels of cyclin E mRNA since 1 cell stage (not shown) and up to 50% epiboly (Fig. 2). The amount of cyclin E mRNA declines at 75% epiboly (Fig. 2) and increases again in adult fish (not shown, Yarden et al., 1994). Methylene blue staining of the blot verified that equal amounts of RNA were loaded in each lane (not shown). Since during the cleavage stage in zebrafish there is no detectable transcription (Kane and Kimmel, 1993) we assume that the cyclin E mRNA found at the 1 cell stage is of maternal origin, and is stable throughout the rapid cell cycles of the cleavage period. We do not know whether novel transcription of the cyclin E gene occurs when zygotic transcription is turned on during MBT.

Cyclin E and Associated Kinase Activity at the Cleavage Stage

In order to study the expression pattern of the cyclin E protein in zebrafish embryos, we have raised polyclonal antibodies to the N-terminus truncated (~40 Kd) bacterially expressed molecule (see Experimental Procedures). The zebrafish cyclin E polyclonal antibodies detected both the bacterially expressed protein as well as a 55 and a 45 Kd protein in zebrafish embryonic extract which was analyzed on a Western blot (Fig. 3, lanes 2 and 1, respectively). Following affinity purification, the 55 Kd protein band became more intense (Fig. 3, lane 5). The pre-absorption of the antiserum on the bacterial antigen, either on the affinity column (Fig. 3, lanes 3 and 4) or on the soluble protein (Fig. 3, lanes 7 and 8), completely abolished its capacity to react with the 55 Kd protein in zebrafish extracts while the 45 Kd protein remained intact (Fig. 3, lanes 3 and 7). THerefore we concluded that the 55 Kd protein is the zebrafish cyclin E and used the affinity-purified antibodies for the subsequent experiments.

As mentioned above, during the cleavage stage in zebrafish the cell cycles are very short (approximately 15 min) and are synchronous (Marrable, 1965). Using Nomarski optics, it is possible to distinguish between mitotic and interphase cells, based on nuclear morphology (Kimmel et al., 1995), and sort interphase and mitotic embryos. Since the maternal cyclin E mRNA levels are constitutive during the cleavage stage, we wondered whether fluctuations in the corresponding protein occur during the early synchronous cell cycles. Western blot analysis of the sorted embryos indicated that similar levels of cyclin E were present in interphase and mitotic embryos (Fig. 4A). The same blot was subsequently probed with monoclonal antibodies against proliferating cell nuclear antigen (PCNA), the total concentration of which was reported to vary minimally during the cell cycle (Waseem and Lane, 1990). Similar levels of the protein were present in interphase and mitotic zebrafish embryos (Fig. 4A), confirming that equal amounts of protein were loaded in each lane. To determine whether cyclin E protein is indeed present during the entire short cell cycle of the cleavage stage, we similarly prepared single embryo extracts at precise time points relative to nuclear membrane breakdown and reformation. Western blot analysis of the sorted embryos indicated that similar levels of cyclin E were present throughout the 7th cell cycle (Fig. 5A). In order to study whether those similar cyclin E levels are due to relatively high stability of the protein or whether cyclin E is synthesized continuously, we microinjected cycloheximide (CHX) into sin-

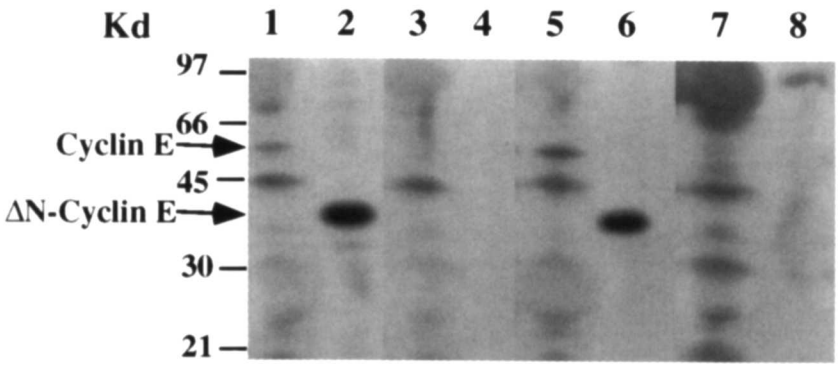

Fig. 3. Characterization of the antibodies to zebrafish cyclin E. Western blot analysis of 150 μg protein of 1 K cell stage zebrafish embryonic extract (**lanes 1, 3, 5,** and **7**) or 10 ng bacterially expressed and purified (see Experimental Procedures) N-terminus truncated cyclin E (**lanes 2, 4, 6,** and **8**). The replica blots were reacted with polyclonal antibodies against cyclin E: crude serum at 1:1,000 dilution (lanes 1 and 2), crude serum which was passed through the cyclin E affinity column at 1:1,000 dilution (lanes 3 and 4), the affinity-purified antibodies at 1:450 dilution (lanes 5 and 6), and the affinity-purified antibodies at 1:450 dilution which were pre-incubated with the bacterially expressed cyclin E (12.5 μg/ml, lanes 7 and 8).

gle embryos at the cleavage stage. Following 10 and 25 min incubation with CHX the cyclin E levels did not change, in contrast to a reduction which was observed in the levels of PCNA in the same embryos, confirming that the protein is stable at this time frame (Fig. 5B).

Cyclin E immunoprecipitates were reported to exhibit histone H1 kinase activity which fluctuates during the mammalian cell cycle with a peak at the G1/S transition (Dulic et al., 1992; Koff et al., 1992). To determine whether during the cleavage stage, which was reported to lack any G1 phase of the cell cycle (Kimmel and Law, 1985), an active cyclin E-CDK complex is constitutively present, we compared the cyclin E-associated H1 kinase activity in interphase and mitotic embryos. As shown in Figure 4B, extracts from both sources were equally effective in phosphorylation of histone H1. The constitutive levels of cyclin E protein and associated H1 kinase activity during the entire cell cycle in cleavage stage zebrafish embryos is in agreement with the constitutive steady-state levels of its mRNA, but in contrast to the periodic expression which occurs in mammalian cells (Dulic et al., 1992).

Cyclin E and Associated Kinase Activity Following the Cleavage Stage

In order to study whether the expression pattern of cyclin E mRNA reflects the expression pattern of the corresponding protein at later stages of embryogenesis, we checked cyclin E protein levels by Western blotting. Protein extracts were prepared from various stages during the first 24 hr of zebrafish embryonic development and reacted with the affinity-purified cyclin E antibodies. In contrast to the reduction in cyclin E mRNA levels, observed at 50–75% epiboly, the cyclin E protein levels did not change significantly throughout epiboly and somitogenesis (Fig. 6A). The same blot was subsequently probed with monoclonal antibodies against PCNA (PC-10), and revealed that similar PCNA protein levels were present throughout the first day of zebrafish embryonic development (Fig. 6A).

To determine whether the high levels of cyclin E protein found in post gastrulating embryos represent high levels of active cyclin E-CDK complexes, we compared the histone H1 kinase activity in cyclin E immunoprecipitates of zebrafish blastula (1K cells, 3 HPF) and 26-somite stage embryos (22 HPF). Cyclin E immunoprecipitates prepared from 1K cell blastula showed high H1 kinase levels (Fig. 6B). In contrast, in the 26-somite stage the cyclin E-associated H1 kinase activity was 10-fold lower (Fig. 6B). Thus, the high levels of cyclin E protein at the 26-somite stage are not reflected by appreciable cyclin E-associated kinase activity (Fig. 6).

We wondered whether the low levels of cyclin E-associated kinase activity in the 26 somite stage, when the cyclin E protein level is high, result from reduced cyclin E-CDK complex formation (Dulic et al., 1992; Koff et al., 1992). Using anti-PSTAIRE specific monoclonal antibodies (reactive with CDK1, CDK2, and CDK3), we showed that the levels of cyclin E-associated 33Kd CDK protein in 1K and 26-somite embryos were essentially identical (Fig. 6B), favoring the possibility that another mechanism other than cyclin-CDK complex formation should be responsible for the reduction observed in the cyclin E-associated H1 kinase activity at the 26-somite stage.

DISCUSSION

The tightly controlled transitions through checkpoints along the mitotic cell cycle are centrally regulated by multiple cyclin molecules and their associated kinases. However, the detailed molecular mechanisms affecting cyclin-CDK activity and the involvement of these and other cell cycle regulators in controlling cellular proliferation during vertebrate embryogenesis are still poorly understood. This issue is of particular

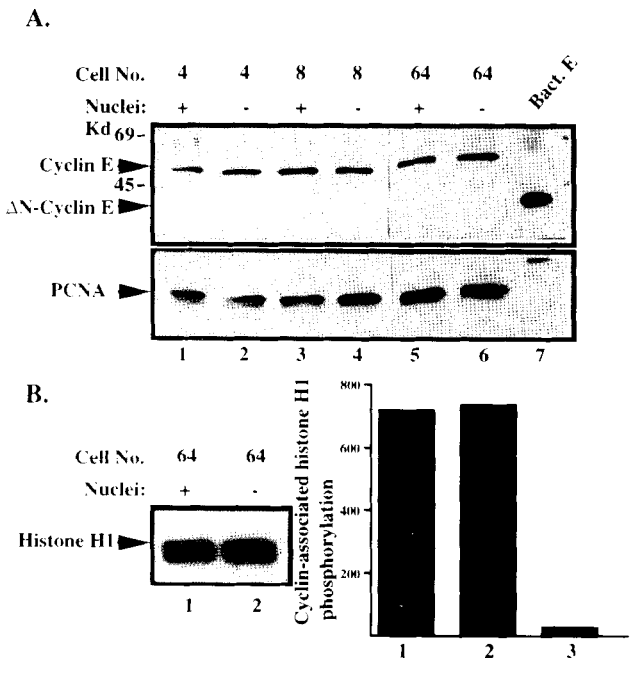

Fig. 4. Immunological detection of the zebrafish cyclin E protein and the associated histone H1 kinase activity in interphase and mitotic cleavage stage embryos. **A:** Western blot analysis of single zebrafish embryos (approximately 40 μg/lane) which were sorted according to presence (+) or absence (−) of nuclear membrane by Nomarski optics as described in Experimental Procedures **(lanes 1–6);** and of the purified, bacterially expressed, N-terminus truncated 40 Kd zebrafish cyclin E following purification, which was used as immunogen **(lane 7).** Single embryos at the 4 cell stage (1 HPF), 8 cell stage (1.25 HPF), and 64 cell stage (2 HPF) were analyzed as indicated on top. The blot was reacted with the affinity-purified anti-zebrafish cyclin E polyclonal antibodies (top), and subsequently with anti PCNA (PC-10) monoclonal antibodies (bottom). **B:** Histone H1 kinase activity of cyclin E immunoprecipitates prepared from 500 μg protein extract corresponding to 12 sorted embryos as in A. The incorporation of ^{32}P into histone H1 was quantitated by Phosphor-Imager (Fuji), and is represented here as Phosphor-Imager arbitrary units. Histograms 1 and 2 correspond to lanes 1 and 2 in the gel to the left, histogram 3 represents the level of histone H1 phosphorylation following immunoprecipitation of the same extract as in lane 1 with rabbit preimmune serum.

importance in studying embryonic morphogenesis which is affected, in a major way, by the coordinated proliferation of its various cell types. Here, we report the identification and characterization of a zebrafish cyclin E homologue. We show that the zebrafish cyclin E is a highly conserved molecule, which is subjected to multiple levels of regulation during embryogenesis.

Characterization of the Zebrafish Cyclin E cDNA

Comparing the sequence of the zebrafish cyclin E to the human homologue pointed to 60% overall identity. The relatively high homology within the G1 cyclin molecule from lower teleosts to mammals may point to their essential function in regulating proliferation. The putative CDK binding site, namely the cyclin box, is the most conserved part in the molecule. The zebrafish CDKs have not been characterized so far, yet we show that a 33 Kd protein, reactive with anti-PSTAIRE antibodies (reactive with CDK1, CDK2, and CDK3 of many species; Yamashita et al., 1991), was detected in zebrafish cyclin E immunoprecipitates (Fig. 6B). We assume that this protein corresponds to the zebrafish CDK bound to cyclin E. It is noteworthy that CDK2 was isolated from goldfish oocytes and was shown to co-immunoprecipitate with a 47 Kd protein which might be the goldfish cyclin E homologue (Hirai et al., 1992).

The zebrafish cyclin E cDNA probe detected a 2.1 Kb mRNA on a Northern blot. This size is similar to that of the cloned cDNA and to the size of the human and the *Xenopus* cyclin E mRNA (Lew et al., 1991; Rempel et al., 1995). Two classes of cyclin E cDNAs which differ in their 5′ ends, were reported to be encoded by the *Drosophila* and the human cyclin E genes (Richardson et al., 1993; Ohtsubo et al., 1995). In zebrafish we detected a single cyclin E mRNA (Fig. 2) as well as a single protein which reacted with our zebrafish cyclin E specific antibodies (Figs. 3, 4, 5, and 6). In addition, using a unique 200 bp fragment from the 5′ end of zebrafish cyclin E cDNA (upstream to the predicted initiator ATG) as a probe for Northern analysis gave similar results to those shown in Figure 2 (data not shown). Therefore, we assume that there is a single type of cyclin E mRNA in zebrafish.

Regulation of Cyclin E in Developing Zebrafish Embryos

The control mechanisms which govern cyclin E expression and activity are very different at various stages of zebrafish embryonic development. During the cleavage stage, the zebrafish cyclin E mRNA, protein, and associated kinase activity are constitutively expressed during a period which lacks a G1 phase, indicating that expression of cyclin E protein and the presence of an enzymatically active cyclin E-CDK may have an additional role during the early embryonic cell cycles, in addition to its established role in the G1/S transition in somatic cells. This stage in zebrafish lacks any novel transcription, and last approximately 3 hr. Therefore, the levels of zebrafish cyclin E mRNA, which is supplied maternally, should stay constant throughout this stage. This is in contrast with the regular cell cycle dependent fluctuations found in cyclin E mRNA, protein, and associated kinase activity levels, previously reported for HeLa cells (Dulic et al., 1992; Lew et al., 1991). In addition, this stability of zebrafish cyclin E mRNA and protein is surprising in view of the short half life of cyclin E mRNA and protein (Sherr, 1993). Similar conclusion concerning high cyclin E mRNA levels were also reported for cleavage stage *Drosophila* (Richardson et al., 1993) and *Xenopus* embryos (Rempel et al., 1995), and in mouse fetal tissues (Damjanov et al., 1994). However, the cyclin E protein was found to be reduced in the *Xenopus* gastrula (Rempel et al., 1995), in contrast to its constitutive levels found in zebrafish. It is possible that different levels of regulation of the cyclin E are present at various time

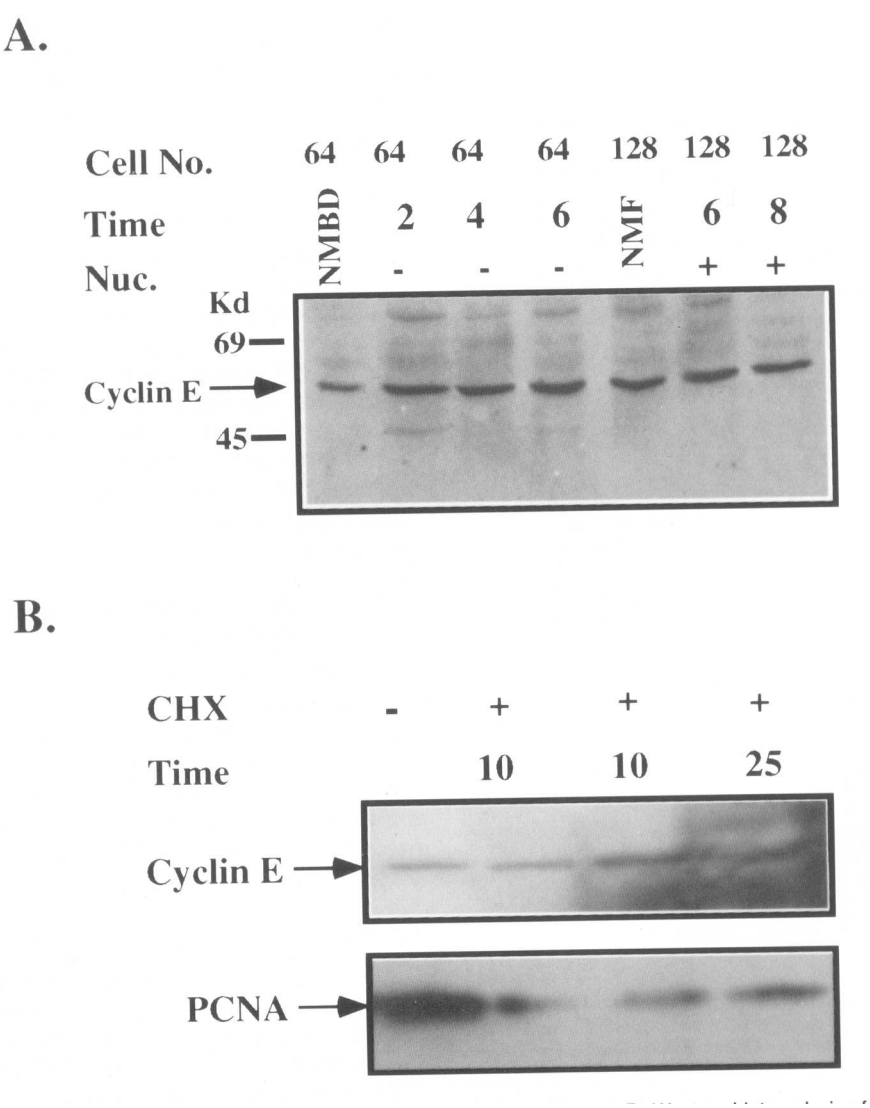

Fig. 5. The zebrafish cyclin E protein is constitutive during the entire cell cycle of cleavage stage embryos. **A:** Western blot analysis of zebrafish embryos which was reacted with the affinity-purified anti-zebrafish cyclin E antibodies. Single embryos were sorted and timed at the exact event of nuclear membrane breakdown (NMBD) and the subsequent 2, 4, and 6 min through mitosis of the 64 cell stage, at the nuclear membrane formation (NMF) and the subsequent 6 and 8 min through interphase of the 128 cell stage. **B:** Western blot analysis of zebrafish embryos which was reacted with the affinity-purified anti-zebrafish cyclin E antibodies (top) and subsequently with anti PCNA monoclonal antibodies (bottom). Single embryos at the 64 cell stage were microinjected with phenol-red alone (−) or with phenol red plus cycloheximide (CHX, 40 μg/ml) and incubated at 28.5°C for 10 or 25 min.

points during the early cell cycles and in fully differentiated adult tissues.

Cyclin E mRNA sharply declines at 75% epiboly and is reduced below detection levels in total zebrafish RNA by the time epiboly reaches completion (Fig. 2). We do not know if novel transcription of cyclin E mRNA occurs at MBT, when zygotic transcription is turned on in the embryo, or whether the cyclin E mRNA which is detected during those time points is still of the maternal pool. During this time the rate of cell division was reported to slow down (Kane et al., 1992; Kane and Kimmel, 1993). The concomitant reduction in cyclin E mRNA levels and cell division rate, implies that elimination of cyclin E might be involved in the inhibition of cell cycle progression and entry into terminal differentiation as previously demonstrated for *Drosophila,* in which the down regulation of cyclin E mRNA was required for arrest of the embryonic cell proliferation (Knoblich et al., 1994).

Examination of the levels of cyclin E-associated CDK and kinase activity during epiboly and segmentation suggests that posttranslational regulation of cyclin E may have a cardinal effect on its activity (Fig. 6). This is manifested by the low mRNA but constitutively high protein levels, the association with CDK (recognized by the PSTAIRE antibodies) but the low kinase activity of

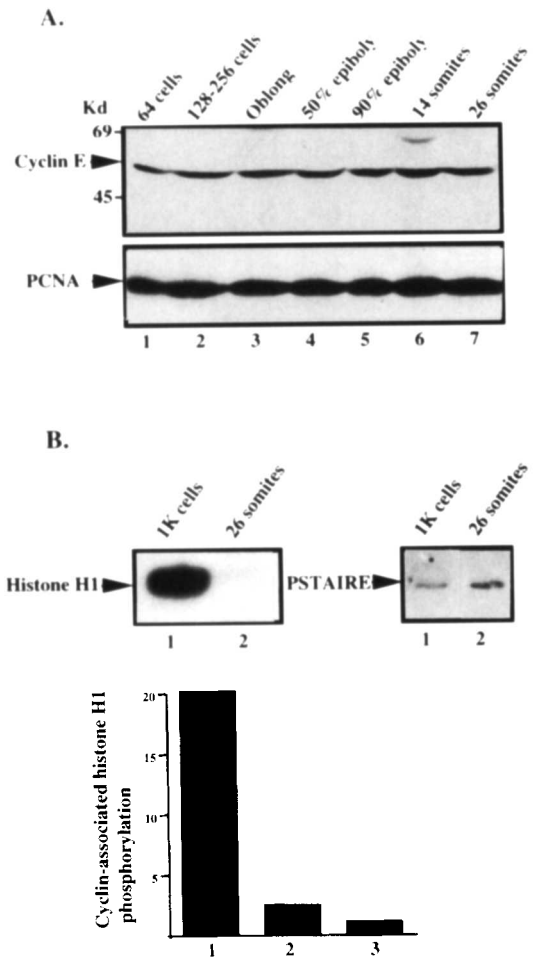

Fig. 6. Immunological detection of the zebrafish cyclin E protein, the associated histone H1 kinase activity, and the formation of cyclin-CDK complexes in developing zebrafish embryos. **A:** Western blot analysis of 150 μg protein per lane, prepared from various developmental stages. The blot was reacted with affinity-purified anti-zebrafish cyclin E polyclonal antibodies (top), and subsequently with anti PCNA (PC-10) monoclonal antibodies (bottom). **B:** Histone H1 kinase activity of anti-cyclin E immunoprecipitates prepared from zebrafish embryos at either 1K cell stage (3 HPF) or 26 somites stage (22 HPF) (left). The incorporation of ^{32}P into histone H1 was quantified by Phosphor-Imager and is represented here as Phosphor-Imager arbitrary units (bottom). Histograms 1 and 2 correspond to lanes 1 and 2 in the gel above, histogram 3 represents the level of histone H1 phosphorylation following immunoprecipitation of the same extract as in lane 1 with rabbit pre-immune serum (bottom). Aliquots from the same extracts used for the histone H1 kinase assay were similarly immunoprecipitated with the anti-cyclin E antibodies and reacted with anti-PSTAIRE monoclonal antibodies on a Western blot (right).

the cyclin E-CDK complex. Such situations in which low cyclin E mRNA and cyclin E-associated kinase activity were observed in cells with high levels of cyclin E-CDK2 complexes, were previously reported in TGF-β treated epithelial cells and in senescent fibroblasts (Dulic et al., 1993; Slingerland et al., 1994). The exact mechanism responsible for such down regulation of the kinase activity is not clear yet but the presence of kinase inhibitor was proposed (Koff et al., 1993; Slingerland et al., 1994). Such inhibitor, if expressed in zebrafish embryos towards the end of the cleavage stage, might induce the addition of the G1 phase to the embryonic cell cycles. Such putative inhibitor(s) might be either one of the known inhibitors of cyclin E-CDK2 complexes like p21, p27, or p57 (reviewed by Peter and Herskowitz, 1994; Sherr and Roberts, 1995), or a new, yet unknown molecule. Another possible inhibitory mechanism may be based on the phosphorylation or dephosphorylation of the regulatory sites on the CDK partner in the complex. For example, down regulation of a CDC25 phosphatase homologue, which is known to activate the cyclin-CDK complex, could exert such effect. Alternatively, the absence of a cyclin-activating-kinase (CAK) homologue may lead to the down regulation of the kinase activity during zebrafish segmentation. Attempts to explore these issues are currently underway.

EXPERIMENTAL PROCEDURES

Maintenance of Fish

Zebrafish were kept at 28.5°C in 10 or 40 liter tanks at a density of up to 1 adult per liter. The water used was charcoal-filtered UV-irradiated double-distilled water, supplemented with salts (Eaton and Farley, 1974). To obtain embryos, 2 wild type female and 4 males were mixed over marbles. Embryos were staged according to Westerfield (1994) and raised in mineral water (Neviot, Israel).

Library Screening

For isolation of the zebrafish cyclin E (*DrcycE*) cDNA, we screened two zebrafish oligo-dT primed cDNA libraries prepared in λZAP (kindly provided by D.J. Grunwald, University of Utah, Salt Lake City, UT). A total of 400,000 phages from a gastrula stage library (6-9 HPF) and 60,000 phages from an adult zebrafish library were screened using *Bam*HI-*Dra*I 240 bp fragment of the genomic clone of *DrcycE* which was previously PCR amplified and cloned (Yarden et al., 1994), as a probe. Hybridization was carried out under stringent conditions (Sambrook et al., 1989). Three clones were isolated and purified by three rounds of hybridization from each library.

RNA Analysis

Total RNA was extracted by a LiCl-Urea protocol (modified from Auffray and Rougeon, 1980). Sixty embryos in their chorions, from each developmental stage, were homogenized in 3M LiCl, 6M Urea, 0.1% SDS, and 10 mM NaAc, pH 5.5; by passing them 10 times through a 21 G needle, and kept on ice for 16–48 hr. Following centrifugation the RNA pellet was washed twice with 4M LiCl in 8M Urea and dissolved in 0.1% SDS in 0.1M NaAc, pH 5.5. The RNA was extracted twice with an equal volume of phenol:chloroform and ethanol precipitated. Ten micrograms total RNA from

each developmental stage were subjected to Northern blot analysis.

Northern blot analysis was performed using standard procedures (Sambrook et al., 1989). RNA was loaded on 1% agarose-formaldehyde gels and run under denaturing conditions. Following transfer onto Magna-NT (MSI, Westboro, MA) membranes, filters were exposed to UV light (1,200 joules, Stratagene UV cross-linker, Stratagene, La Jolla, CA) for 1 min and stained with methylene blue (Sambrook et al., 1989) to visualize the rRNA as a standard for verifying transfer and comparing the relative quantity of RNA in the various samples. Hybridization to random-primed probes was carried out under stringent conditions (Sambrook et al., 1989). The gel-purified probe used was *Bam*HI-*Eco*RI fragment of *DrcycE* cDNA.

Preparation of Specific Antibodies and Protein Purification

The pET8c (Studier et al., 1990) was used to bacterially express an N-terminus truncated (71 aa) *DrcycE*. A 1.7 Kb *Bam*HI fragment of *DrcycE* cDNA was inserted into the *Bam*HI site of pET8c and formed an in-frame fusion protein containing 11 aa of the pET8c vector to the N-terminus of the truncated *DrcycE*, to create a plasmid designated pET8c-cycE. This plasmid was introduced into pLYS-S DE3 cells (Studier et al., 1990) and the protein expressed following 0.5 mM IPTG induction and analyzed on 10% PAGE followed by Coomassie-blue staining. A prominent band of the expected molecular weight (40 Kd) of the truncated *DrcycE* was visualized.

The IPTG-induced protein was purified from inclusion bodies (Sambrook et al., 1989). Briefly, cells from 500 ml of IPTG-induced culture at $A_{600}=0.6$, were lysed at room temperature in NET (100 mM NaCl, 1 mM EDTA, 50 mM Tris, pH 8.0) containing 1 mM phenylmethylsulfonyl fluoride and lysozyme (1 mg/ml). All subsequent steps were carried out at 4°C. Following centrifugation at 12,000*g* the pellet was resuspended in NET containing 0.1% Na-deoxycholate. DNaseI (10 μg/ml) and $MgCl_2$ (8 mM) were added for 45 min or until viscosity decreased. Pellets were washed with NET followed by 3 washes in 1 M Urea resuspended in 8 M Urea and kept on ice for 4 hr. Following centrifugation, the supernatant, containing the purified desired proteins, was dialyzed against PBS. A series of 3 injections into rabbits every 10 days of 150 μg of the purified cyclin E protein were performed. The antiserum was affinity purified on cyclin E affinity column prepared according to Wilchek (Wilchek et al., 1994).

Protein Analysis

Proteins were extracted from zebrafish embryos in RIPA buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 5 mM EDTA, 1% Na-deoxycholate, 1% Triton X-100) containing 1 mM PMSF, 10 μg/ml leupeptin, 10 μg/ml pepsin, 20 μg/ml aprotonin, 10 μM NaPPi, 50 μM NaF, and 0.5 mM $NaVO_4$. For the preparation of the developmental Western, sixty embryos from each developmental stage were sonicated in 100 μl of RIPA buffer and microfuged for 10 min. The clear supernatant protein extract was loaded (150 μg per lane) onto 10% PAGE. For single embryo analysis, embryos were placed in mineral water in depression-slides and observed under Nomarski optics using a 40X/N.A.0.75-water immersion objective (Zeiss, Oberkochen, Germany). The embryos were sorted for the presence or absence of nuclei, and once staged, they were placed in 40 μl RIPA buffer at 4°C, sonicated and loaded onto 10% PAGE (1 embryo equivalent per lane, approximately 40 μg). For the cycloheximide experiments, 5 nl of cycloheximide (400 μg/ml in 0.5% Phenol-red) were microinjected into the yolk under a stero-scope using an Eppendorf automatic microinjector. Blotting was carried out for 1 hr in 50 mM Tris, 50 mM glycine, and 1 mM $MgCl_2$ at 200 mA onto Hybond-C (Amersham, Buckinghamshire, UK) at 0–4°C. The filters were incubated with either anti-*DrcycE* affinity-purified polyclonal antibodies (at 1:200 dilution), or anti-PCNA monoclonal antibodies (at 1:3,000 dilution, clone PC-10, Sigma ImmunoChemicals, Rehovot, Israel). Detection of bands was carried out using peroxidase-coupled goat anti-rabbit antibodies and the ECL system (Amersham).

For immunoprecipitation, 500 μg of zebrafish protein extract were incubated with the affinity-purified cyclin E polyclonal antibodies, followed by precipitation with protein A-Sepharose (CL-4B, Pharmacia, Uppsala, Sweden). The Histone H1 kinase assay was carried out as described by (Resnitzky et al., 1994). The background level of possible non-specific kinase activity was verified by preincubation of the affinity-purified cyclin E polyclonal antibodies with the bacterially expressed cyclin E (12.5 μg/ml). For reaction with the anti-PSTAIRE monoclonal antibodies (Yamashita et al., 1991), the cyclin E immunoprecipitates were washed several times with RIPA buffer and heated 10 min at 37°C in sample buffer before loading onto 10% PAGE and blotted as above. The blot was incubated with the anti-PSTAIRE monoclonal antibodies at 1:500 dilution.

ACKNOWLEDGMENTS

We thank D.J. Grunwald, R. Riggleman, and K. Helde for the zebrafish cDNA libraries and S.I. Reed for cyclin E oligonucleotides. We thank D. Aron, T. Miron, and D. Salomon for technical assistance. We also thank N. Tiefenbrun and D. Resnitzky for helpful discussions and suggestions during the course of this work. This study was supported by grants from the Council for Tobacco Research-U.S.A., the Leo and Julia Forchheimer Center for Molecular Genetics at the Weizmann Institute of Science, and the Minna James Heineman Foundation. B.G. holds the E. Neter Professorial Chair in Cell and Tumor Biology.

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