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# Cyclin $E_T$, a new splice variant of human cyclin E with a unique expression pattern during cell cycle progression and differentiation

**Dominik Mumberg, Maresa Wick, Christiane Bürger, Kirsten Haas, Martin Funk and Rolf Müller***

Institut für Molekularbiologie und Tumorforschung (IMT), Philipps-Universität Marburg, Emil-Mannkopff-Strasse 2, D-35033 Marburg, Germany

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## ABSTRACT

**Cyclin E is the regulatory subunit of the cdc2-related protein kinase cdk2 and is a rate limiting factor for the entry into S phase. To date, cyclin E is the only cyclin for which alternative splicing has been described. We report here the isolation of a new splice variant of cyclin E, termed cyclin $E_T$, which has an internal deletion of 45 amino acids compared with the full-length cyclin E protein. Even though cyclin $E_T$ contains an intact cyclin box, it is unable to complement a triple *cln* mutant strain of *Saccharomyces cerevisiae* or to interfere with rescue by cyclin E, indicating that an intact cyclin box is functionally insufficient. The expression pattern of cyclin $E_T$ during cell cycle entry, progression and differentiation differs from that of cyclin E. Thus, $E_T$ expression precedes that of the other isoforms during the $G_0 \rightarrow S$ progression; it shows a sharp peak in early $G_1$ in cells released from a mitotic block and is strongly down-regulated in terminally differentiated myeloid cells. These observations point to different functions for cyclin $E_T$ and E and show for the first time that the alternative splicing of cyclin E is a regulated mechanism governed by the cell cycle and differentiation.**

## INTRODUCTION

Progression through the $G_1$ phase of the cell cycle and entry into S phase in mammalian cells are controlled by a number of proteins, including the cdc2-related kinases cdk2, cdk4 and cdk6, their regulatory subunits, the D and E type cyclins, different inhibitors of cdks such as p15/INK4B, p16/INK4/MTS, p21/WAF1/CDI2/CIP1/SDI1/PIC1 and p27/KIP, the retinoblastoma suppressor protein pRB and its kins p107 and p130 and transcription factors of the E2F/DRTF family (for recent reviews see 1–7). To date, four cyclins (D1, D2, D3 and E) have been described whose functions seem to be associated with, and required for, $G_1$ progression (8–11). The D-type cyclins have been shown to associate with different proteins, such as cdk2, cdk4, cdk6, pRB, the DNA polymerase δ subunit PCNA and other gene products (12–18; for reviews see 1,3,4,6,7,19). Complexes between D-type cyclins and pRB have been detected in immunoprecipitates and cdk4-mediated pRB phosphorylation has been observed both *in vitro*, in insect cells over-expressing cyclin D, cdk4 and pRB and in cyclin D complexes isolated from mammalian cells (14,15). Cyclin E, on the other hand, appears to be less promiscuous. It is well established now that cyclin E forms complexes specifically with cdk2 and activates its serine-threonine kinase activity shortly prior to entry into S phase (20,21). This observation suggests that the cyclin E–cdk2 complexes might be directly involved in regulating the $G_1$/S transition. This is supported by the finding that microinjection of cdk2- or cyclin E-specific antibodies results in a $G_1$ block (22,23). In addition, cells transformed by a cyclin E-expressing retrovirus show a decreased cell size, a decreased requirement for growth factors and a shorter $G_1$ phase of the cell cycle (24). In addition, the inducible expression of cyclin E from a tetracycline-regulatable vector has been shown to lead to a shortening of the $G_1$ phase (25,26).

In contrast to cyclin D, which represents a family of related genes (11), only a single gene for cyclin E has been detected (23,27). On the other hand, cyclin E is the only cyclin gene for which alternative splicing leading to a structurally different protein has been described. We have previously reported the existence of a 43 kDa splice variant of human cyclin E, termed cyclin $E_S$, which lacks 49 amino acids within the cyclin box compared with the known full-length 48 kDa cyclin E. Cyclin $E_S$ is expressed at ~10% of the level of full-length cyclin E in all cell lines analysed. The two cyclin E forms functionally differ in that cyclin E, but not cyclin $E_S$, is able to complex with cdk2, to activate the histone H1, pRb and p107 *in vitro* kinase activity of cdk2 and to rescue a triple *cln* mutation in *Saccharomyces cerevisiae*. These findings also indicate that the cyclin box in cyclin E is required for the interaction with cdk2 and the other functions described above, but the biological function of cyclin $E_S$ remains obscure. In the present study we describe the identification and analysis of a new splice variant of cyclin E RNA, termed cyclin $E_T$, which has an internal deletion of 45 amino acids relative to the full-length cyclin E protein. Although this deletion leaves the cyclin box intact, cyclin $E_T$ is unable to function as a $G_1$ cyclin in yeast, pointing to a crucial functional

*To whom correspondence should be addressed. Tel.: +49 6421 28 6236; Fax: +49 6421 28 8923; Email: mueller@imt.uni-marburg.de

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role for sequences ouside the cyclin box. We also show that cyclin $E_T$ expression during the cell cycle precedes up-regulation of the other isoforms and dramatically decreases in terminally differentiated myeloid cells. These observations suggest that cyclin $E_T$ and E serve different functions. In addition, this is the first report of an alternative RNA processing event that is controlled by cell cycle progression and differentiation.

## MATERIALS AND METHODS

### Cell culture

WI-38 cells (28), obtained from the American Type Culture Collection (ATCC), and HaCat cells (29) were cultured in Dulbecco–Vogt modified Eagle's minimum essential medium (DMEM) supplemented with 10% fetal calf serum (FCS). WI-38 cells were seeded at a density of $10^4$ cells/$cm^2$ 24 h prior to starvation in serum-free medium for 72 h and stimulated with 10% FCS for the indicated times. In some experiments transcription was inhibited by the addition of actinomycin D (5 µg/ml; Sigma) to the medium. HaCat cells were synchronized in mitosis by a combined hydroxyurea/nocodazole block. After 14 h incubation with 0.1 M hydroxyurea (Sigma) cells were cultured for 9 h in fresh medium, followed by another 5 h incubation in the presence of 30 ng/ml nocodazole (Sigma). The mitotic cells were subsequently detached from the plate by gentle shaking, washed twice with phosphate-buffered saline (PBS) and replated into fresh medium. HL-60 cells were grown in RPMI 1640 supplemented with 10% FCS. Differentiation of HL-60 cells by $10^{-7}$ M 12-*O*-tetradecanoylphorbol-13-acetate (TPA; Sigma) or $10^{-6}$ M retinoic acid (RA; Sigma) was induced as described (30,31), except that the TPA was removed after 30 min. Native granulocytes and monocytes were isolated from peripheral blood samples by Ficoll density gradient centrifugation as previously described (32).

### FACS analysis

For FACS analysis, cells were washed once with PBS and fixed in ice-cold 75% ethanol overnight at 4°C. After washing once with PBS, the fixed cells were stained for 15 min in Hoechst 33258 staining buffer (100 mM Tris, pH 7.4, 154 mM NaCl, 1 mM $CaCl_2$, 0.5 mM $MgCl_2$, 0.1% v/v Nonidet P-40, 0.2% w/v bovine serum albumin, 2 µg/ml Hoechst 33258). Stained cells were analysed in a FACS-STAR Plus (Becton-Dickinson) using UV laser excitation at 325 nm. The fluorescence was amplified linearly. Cell cycle distributions were calculated using the LYSIS II program (Becton Dickinson).

### RNA isolation and analysis by reverse transcriptase PCR (RT–PCR)

RNA isolation, reverse transcription into cDNA and PCR analysis were performed as described (33). The RT–PCR established in our laboratory (33) yields results that are basically identical to the data obtained by Northern blotting, as previously shown by a direct comparison (34). The experimental strategy included the following precautions: (i) the number of PCR cycles was kept low in order to obtain a linear relationship between the levels of specific input RNA and PCR product, which was achieved by the incorporation of radioactive precursor nucleotides and evaluation by β-radiation scanning using a Molecular Dynamics PhosphorImager; (ii) all results were standardized using the signal obtained with GAPDH or β-actin, whose expression is independent of cell proliferation or differentiation; (iii) all experiments were performed with at least two independent RNA and cDNA preparations and yielded results that showed <20% deviation after standardization; (iv) to control for DNA contamination, primers were designed that spanned at least one exon–intron boundary. The following cyclin E primer pairs were used for PCR amplification (8): 5′-Primer, 5′-GCGGATCCTGGGCAAATAGAGAGGAAGTCTGG-3′ (342–365); 3′-Primer, 5′-GGCTCGAGCGAGAAATGATACAAGGCCG-3′ (936–917). Underlined sequences indicate restriction sites used for cloning of PCR fragments.

### *In vitro* translation and immunoprecipitation

The open reading frames of the three cyclin E isoforms were cloned into the eukaryotic expression vector pBK-RSV (Stratagene) and N-terminally fused to a three times repeated nine amino acid 12CA5 hemagglutinin (HA) epitope (35). Expression of the tagged cyclin E proteins was controlled by *in vitro* translation using the Promega TNT T3 reticulocyte lysate system following the instructions of the manufacturer. Detection of *in vitro* translated HA–cyclin E proteins was monitored by immunoprecipitation using a monoclonal α-HA antibody (Babco, Berkeley, CA). Four microlitres of *in vitro* translated HA–cyclin E proteins were incubated with 4 µl affinity purified α-HA antibody for 30 min at 4°C followed by incubation for 20 min with 40 µl protein G–Sepharose 4 Fast Flow (Pharmacia). Immune complexes were centrifuged and pellets were washed three times in IPB buffer (50 mM Tris–HCl, pH 7.6, 2 mM NaCl, 0.5% w/v NP-40, 0.02% aprotinin, 2 mM PMSF, 1 mM DTT), resuspended and boiled in SDS sample buffer.

### Yeast techniques

The yeast strain DL1 (MATα, *ade1*, *his2*, *leu2-3*, *112*, *trp1-1a*, *ura3*, *cln1*::*TRP1*, *cln2*, *cln3*, *leu2*::*GAL1-CLN2*) was kindly provided by Steve Reed (Scribbs Research Institute, La Jolla, CA). Yeast cells were grown on either YPGR (1% yeast extract, 2% Bactopeptone, 3% galactose, 3% raffinose) or $S_{GR}$ medium (36) with or without 1 mM methionine. For repression of the GAL1 promoter 2% glucose was added. All *S.cerevisiae* expression vectors used have been described (37,38) and transformations were carried out according to Gietz *et al.* (39).

## RESULTS AND DISCUSSION

### Detection of a new isoform of cyclin E

We have recently described an alternatively spliced variant of human cyclin E, which we termed cyclin $E_S$ (27). A detailed analysis of human cyclin $E_S$ expression by RT–PCR with specific primer pairs revealed the existence of an additional cyclin E isoform in WI-38 lung fibroblasts that is only slightly larger than cyclin $E_S$ (Fig. 1). Expression of this new isoform of human cyclin E, which we have called cyclin $E_T$ (for third isoform of cyclin E), was also detected in a variety of other human cell lines, such as promyelocytic HL-60 cells, HaCaT keratinocytes and the cervical carcinoma HeLa cell line, and is therefore obviously not restricted to a certain cell type. In all these cell lines the expression

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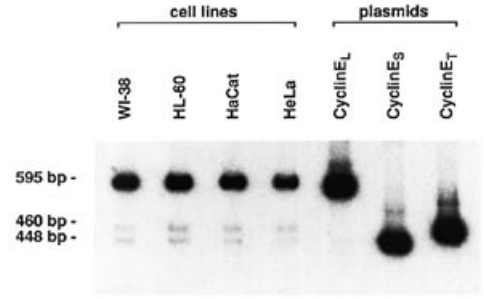


**Figure 1.** Detection of a novel isoform of cyclin E. RNA from the indicated human cell lines was analysed by RT–PCR. As a control, PCR reactions were also performed with plasmids containing the respective cloned PCR-generated fragments.

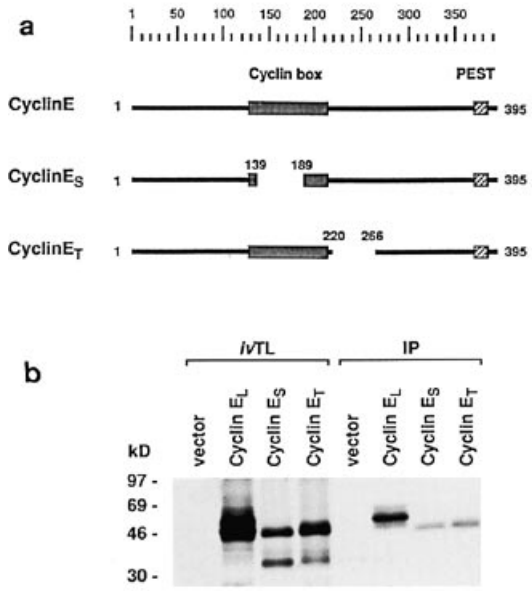


**Figure 2.** (**a**) Schematic representation of the three human cyclin E isoforms. Numbers refer to amino acid positions in the cyclin E protein. (**b**) Detection of HA–cyclin E fusion proteins after *in vitro* translation of the indicated cDNAs (*iv*TL) and subsequent immunoprecipitation (IP) using a monoclonal α-HA antibody. Vector, expression vector without cDNA.

of cyclin $E_T$ was similar to that of cyclin $E_S$, although there may be subtle cell type-specific differences (Fig. 1).

## Characterization of cyclin $E_T$

In order to obtain more information about the nature of cyclin $E_T$, the corresponding cDNA was cloned via cyclin E-specific PCR primers. Subsequent sequence analysis showed that the difference in size between cyclin $E_T$ and cyclin $E_S$ is only 12 bp. Despite their nearly identical size, however, both encoded proteins clearly differ in their molecular structure (Fig. 2a). Whereas cyclin $E_S$ has an internal deletion of 49 amino acids within the cyclin box (27), cyclin $E_T$ contains an intact cyclin box but has undergone an in-frame deletion of 45 amino acids immediately C-terminal of the cyclin box (amino acids 221–265 of the full-length cyclin E protein, subsequently referred to as

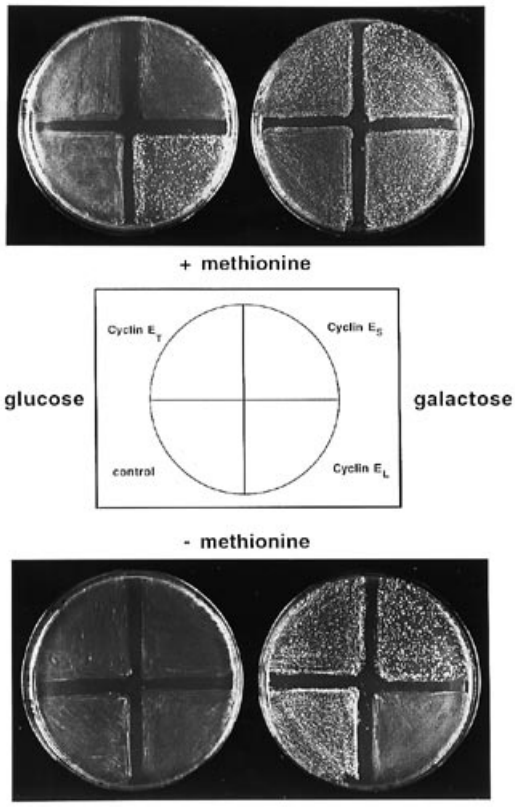


**Figure 3.** Rescue of *S.cerevisiae* triple *cln* mutant strains by cyclin $E_L$ but not by cyclin $E_T$ or $E_S$. The yeast expression vector pRS426Met25 (37) containing the indicated cDNAs was transformed into the yeast strain DL1 (9). Transformants were directly transferred onto selective agar plates containing either glucose (left) or galactose (right) in the presence (upper) or absence (lower) of 1 mM methionine. All plates were incubated for 3 days at 30°C. Control, expression vector without cDNA.

cyclin $E_L$, for long isoform of cyclin E). Apart from this deletion of 135 bp the nucleotide sequence of the cyclin $E_T$ cDNA is identical to that of cyclin $E_L$ (data not shown; 8,9). Cyclin E is a single copy gene in humans (23,27) and the nucleotide sequences around positions 717 and 853 (8) show significant homologies to splice donor and acceptor consensus sites:

consensus: 5′-AG/GT-----AG/G-3′
cyclin $E_T$: 5′-$_{716}$AG/GC-----AG/C$_{853}$-3′.

These results therefore strongly suggest that cyclin $E_T$ is generated by alternative splicing of the primary cyclin E transcript, as is cyclin $E_S$.

In order to verify that the cyclin $E_T$ cDNA has the potential to code for a protein, all three cyclin E isoforms were cloned into the vector pSK-II-Bluescript (Stratagene) and were subsequently *in vitro* transcribed and translated. To allow for specific detection of the respective cyclin E protein products by immunoprecipitation, the proteins were tagged with a 3×HA epitope from influenza virus (35). As can be seen in Figure 2b, the HA–cyclin $E_T$ cDNA was efficiently translated into a protein of ~46 kDa in size that was specifically immunoprecipitated by a monoclonal α-HA antibody (Babco). The faster migrating bands visible in the *in vitro* translation most likely represent degradation products. Considering the size of the HA tag (3×9 amino acids), the molecular weight

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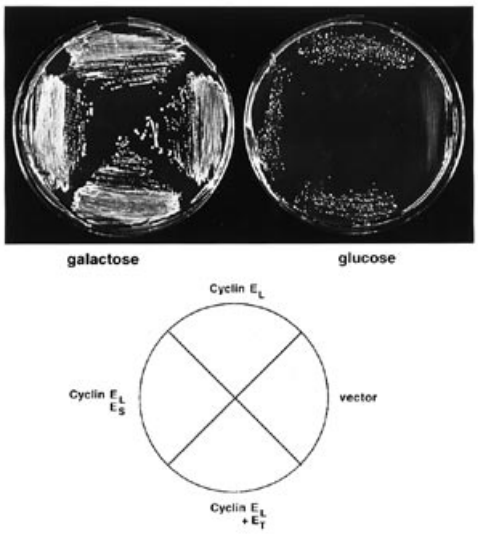


**Figure 4.** Cyclins $E_T$ and $E_S$ do not interfere with the rescue of triple *cln* mutant yeast strains by cyclin $E_L$. The yeast expression vector pRS416ADHcyclin $E_L$ (38) was co-transformed with either pRS415GPD, pRS415GPDcyclin $E_T$ or pRS415GPDcyclin $E_S$ into the yeast strain DL1 (9). Transformants were transferred onto selective agar plates containing either galactose or glucose and were incubated for 3 days at 30°C. Vector, pRS416ADH co-transformed with pRS415GPD.

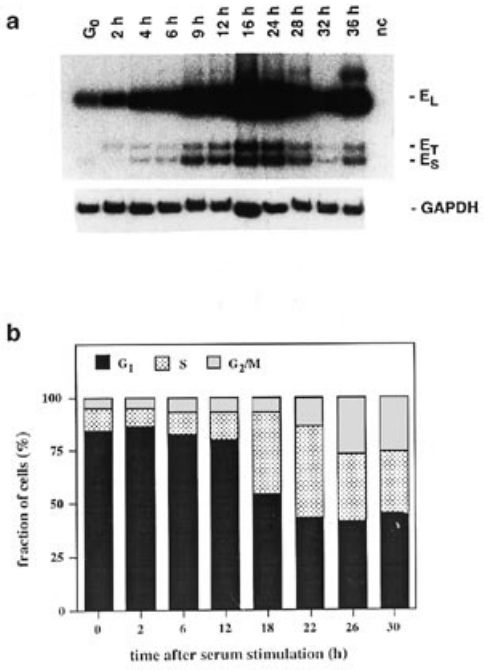


**Figure 5.** (**a**) Expression of cyclin E splice variants in serum-stimulated WI-38 human fibroblasts synchronized in $G_0$ by serum deprivation. RNA was isolated at the indicated time points after serum stimulation and analysed by RT-PCR. The GAPDH signal was used as an internal standard. nc, control PCR without cDNA input. (**b**) FACS analysis of cell cycle distribution at the indicated time points after serum stimulation of WI-38 human fibroblasts.

of the cyclin $E_T$ protein is ~43 kDa and therefore practically identical to that of cyclin $E_S$ (27). In agreement with this conclusion, the HA–cyclin $E_S$ translational product showed the same electrophoretic mobility as HA–cyclin $E_T$ (Fig. 2b). Consequently, the detection of cyclin $E_T$ protein expression *in vivo* has to await isoform-specific anti-cyclin E antibodies.

## Cyclin $E_T$ is unable to rescue yeast triple *cln* mutants

Cyclin E was originally discovered by virtue of its ability to complement a $G_1$ cyclin deficiency in *S.cerevisiae* (8,9). In order to analyse the functional properties of cyclin $E_T$, its cDNA as well as that of cyclin $E_L$ and $E_S$ were cloned into the yeast expression vector pRS426Met25 (37) and subsequently transformed into the *S.cerevisiae* strain DL1 (kindly provided by Dr S.Reed, Scribbs Research Institute, La Jolla, CA). DL1 yeast cells lack the major $G_1$ cyclins CLN1, -2 and -3, but harbour an exogenous CLN2 gene under the control of the inducible GAL1 promoter. As a consequence these cells can only grow in the presence of galactose, but not glucose. Accordingly, as can be seen in the upper panel of Figure 3, all transformants formed colonies on galactose-containing agar plates. In the presence of glucose, however, only cyclin $E_L$-expressing DL1 cells could grow, whereas cyclin $E_T$ and cyclin $E_S$ were unable to rescue the triple *cln* deficiency. It has been shown that strong over-expression of cyclin $E_L$ is toxic in *S.cerevisiae* (27). Therefore our experiments were performed in the presence of 1 mM methionine. Under those conditions the Met25 promoter is repressed and gives rise to low levels of expression (37). However, to exclude the possibility that the failure of cyclin $E_T$ and $E_S$ to rescue triple *cln* mutant yeast cells was due to an insufficient expression level, the same experiment was carried out in the absence of methionine, which led to high levels of expression for all three cyclin E variants ($E_L$, $E_S$ and $E_T$), as determined by Westen blot analysis (27; data not shown). However, even this strong over-expression of cyclin $E_T$ or cyclin $E_S$ was insufficient to enable the DL1 cells to proliferate in the presence of glucose (Fig. 3, lower panel). Therefore, it can be concluded that cyclin $E_T$ is unable to complement a $G_1$ cyclin deficiency in *S.cerevisiae*. Furthermore, these results clearly show that the presence of an intact cyclin box is insufficient for cyclin E to function as a $G_1$ cyclin in *S.cerevisiae*. This observation suggests that cyclin $E_T$ might be unable to interact with cdk partners. This notion was indeed confirmed by a yeast two-hybrid experiment. While a lex A–cyclin $E_L$ hybrid protein showed efficient binding to a VP16–cdk2 fusion protein, there was no evidence for any interaction of the corresponding lex A–cyclin $E_T$ hybrid and the VP16–cdk2 protein in the same assay (data not shown).

As expected, cyclin $E_L$-over-expressing cells were unable to form colonies on either galactose- or glucose-containing agar plates in the absence of methionine. Although the molecular mechanisms underlying this cyclin E-mediated toxicity are unclear at present, the fact that neither cyclin $E_T$ nor $E_S$ showed any toxic effects under those conditions points toward a correlation between the ability of cyclin E to rescue a triple *cln* mutant and its toxicity under conditions of strong over-expression.

## Cyclin $E_T$ cannot antagonize cyclin $E_L$ function in yeast

Since the cyclin box is the only domain shared by all known cyclin proteins, this domain has been implicated in the binding of cyclins to their catalytic cdk partners. Indeed, the cyclin box of cyclin A is not only required for binding of cdc2 and cdk2 (40,41), but even seems to be sufficient (41). As cyclin $E_T$ is unable to function as a $G_1$ cyclin in *S.cerevisiae* but contains an

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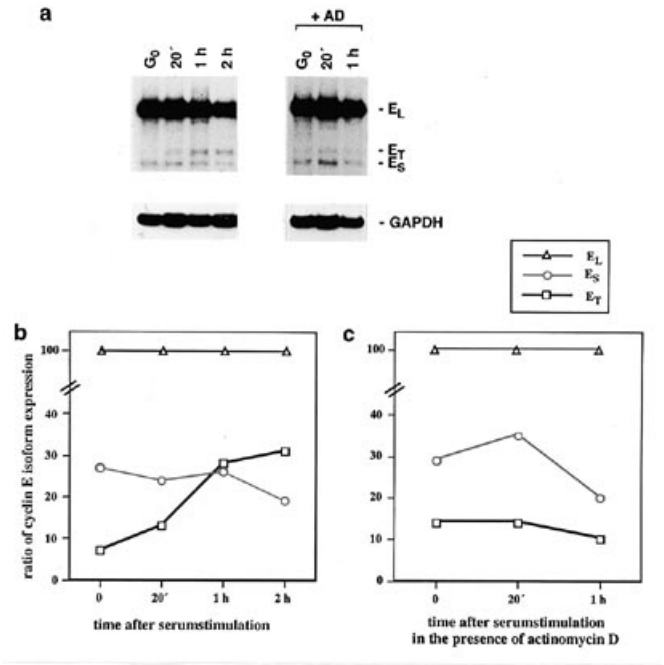


**Figure 6.** Short-term kinetics of the expression of cyclin E splice variants in serum-stimulated WI-38 human fibroblasts synchronized in $G_0$ by serum deprivation. (**a**) RNA was isolated at the indicated time points after serum stimulation in the absence or presence of the RNA polymerase inhibitor actinomycin D (+AD) and analysed by RT–PCR. The GAPDH signal was used as an internal standard. (**b** and **c**) Ratio of cyclin $E_T$ and $E_S$ expression relative to cyclin $E_L$ after serum stimulation in the absence (b) or presence (c) of actinomycin D. Results from (a) were quantitatively evaluated by β-radiation scanning and standardized for GAPDH expression. The expression levels of cyclin $E_T$ and $E_S$ at any given time point are expressed as a percentage of the corresponding level of cyclin $E_L$ expression. See Materials and Methods for details on the fidelity of the RT–PCR analyses performed in the present study.

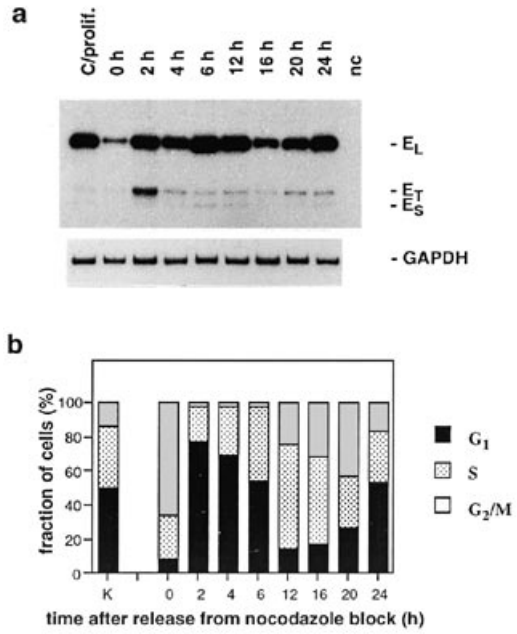


**Figure 7.** (**a**) Expression of cyclin E splice variants in synchronized human HaCat cells after release from a nocodazole-induced cell cycle arrest. Normally proliferating HaCat cells were synchronized by successive hydroxyurea/nocodazole blocks (as described in Materials and Methods) and RNA was isolated at the indicated time points after release from the block. Expression analysis was performed via RT–PCR and the GAPDH signal was used as an internal standard. C/prolif., normally proliferating HaCat cells; nc, control PCR without cDNA input. (**b**) FACS analysis of cell cycle distribution at the indicated time points of human HaCat cells after release from a nocodazole-induced cell cycle arrest.

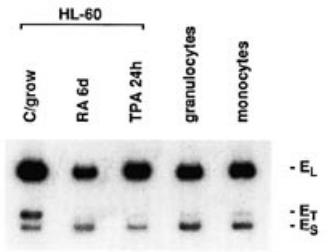


**Figure 8.** Expression of cyclin E splice variants in differentiated haematopoietic cells. RNA was isolated from normally growing HL-60 cells (C/grow), from terminally differentiated HL-60 cells 6 days after stimulation with RA or 24 h after stimulation with TPA and from native granulocytes and monocytes derived from peripheral blood samples. Expression analysis was performed via RT-PCR.

intact cyclin box, it might be able to interfere with the function of cyclin $E_L$ in a *trans*-dominant negative manner. To test this hypothesis, cyclin $E_T$ was co-expressed with cyclin $E_L$ in DL1 yeast cells (Fig. 4). To ensure strong over-expression of cyclin $E_T$ relative to cyclin $E_L$ in these experiments, cyclin $E_T$ was expressed from the GPD promoter (pRS415GPD) whereas cyclin $E_L$ expression was under the control of the ADH promoter (pRS416ADH), whose transcriptional activity is ~70-fold weaker than that of the GPD promoter (38). Despite this over-expression, cyclin $E_T$ (or cyclin $E_S$) was unable to interfere with the ability of cyclin $E_L$ to complement the $G_1$ cyclin deficiency of the DL1 yeast cells. In summary, these results show that cyclin $E_T$ is neither able to act as a $G_1$ cyclin in *S.cerevisiae* nor to antagonize the rescue of triple *cln* yeast mutants by cyclin $E_L$.

## Expression of cyclin E isoforms is differentially regulated during cell cycle entry and progression

The expression pattern of cyclin $E_S$ during serum stimulation of WI-38 fibroblasts and differentiation of promyelocytic HL-60 cells is identical to that of cyclin $E_L$ (27,34), indicating that at least under those conditions the alternative splicing of cyclin $E_S$ is not regulated. In order to investigate whether the expression of cyclin $E_T$ may follow a different pattern, we studied the expression of cyclin E during the cell cycle. In a first set of experiments, cyclin $E_T$ expression was analysed during the $G_0 \rightarrow S$ progression of serum-stimulated WI-38 fibroblasts (Fig. 5). In agreement with previous studies (9,27), the expression of all three cyclin E mRNA isoforms was low in $G_0$ (Fig. 5a), was induced after 9 h, reached peak levels at 16 h, i.e. around entry into S phase (Fig. 5b), and declined as the cells proceeded through $G_2$ and M. Although the overall expression pattern of all three cyclin E isoforms was similar, induction of cyclin $E_T$ was already detectable 2 h after serum stimulation. In order to verify and extend this observation, the 0–2 h time interval was analysed in more detail. As can be seen in Figure 6, an increase in the level of cyclin $E_T$ RNA was detectable as early as 20 min post-stimulation. After 2 h a 4-fold induction relative to cyclin $E_L$ was

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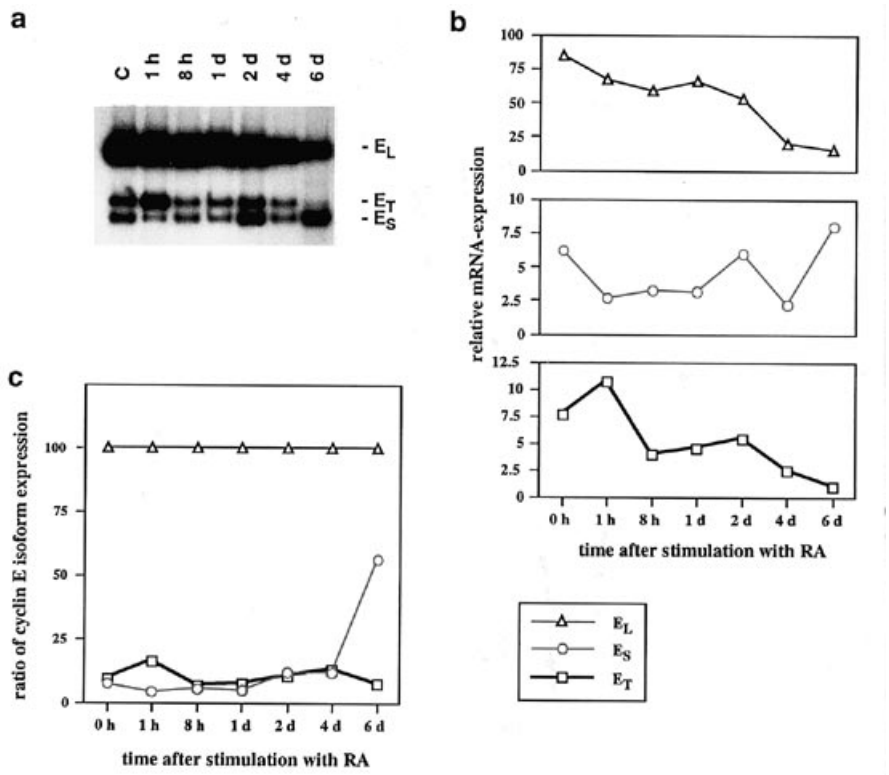


**Figure 9.** Expression of cyclin E splice variants in differentiating HL-60 cells after stimulation with RA. (**a**) Human HL-60 cells were induced to differentiate into granulocyte-like cells by stimulation with RA. RNA was isolated at the indicated time points after stimulation and analysed via RT–PCR. C, normally proliferating HL-60 cells. (**b**) Quantitative evaluation of the results obtained in (a) and standardization for β-actin expression. The lowest expression level (cyclin $E_T$/6 days) was assigned the arbitrary value of 1. (**c**) Ratio of cyclin $E_T$ and $E_S$ expression relative to cyclin $E_L$. The expression levels of cyclin $E_T$ and $E_S$ are expressed as a percentage of the corresponding level of cyclin $E_L$ expression.

observed. In contrast, the expression of cyclin $E_S$ relative to cyclin $E_L$ remained constant during this period. The induction of cyclin $E_T$ was inhibited in the presence of actinomycin D (Fig. 6c), indicating that this effect was dependent on *de novo* synthesis of $E_T$ mRNA and not simply due to increased stability of the cyclin $E_T$ transcript. These results show that the alternative splicing of human cyclin E RNA is not a constitutive process, but instead is regulated during $G_0 \rightarrow S$ progression.

To address the question of whether the alternative splicing of cyclin $E_T$ is also regulated during normal cell cycle progression, HaCat cells were synchronized at $G_2$/M by successive hydroxyurea and nocodazole blocks. After release from the cell cycle arrest, cyclin E expression was monitored by RT–PCR. As shown in Figure 7b, the synchronized cells underwent mitosis within 2 h after release from the $G_2$/M block. After 12 h most cells were in S phase and entered the next cell cycle between 20 and 24 h with a somewhat decreased synchrony. As expected, expression of cyclin $E_L$ was low in the $G_2$/M-arrested HaCat cells (Fig. 7a), reached peak levels around the $G_1 \rightarrow S$-transition (4-fold induction after 6 h), declined as cells entered the $G_2$ and M phases and was induced again in the subsequent $G_1$ phase. Whereas the expression of cyclin $E_S$ paralleled that of cyclin $E_L$, cyclin $E_T$ showed a clearly different pattern. Two hours after release from the $G_2$/M arrest, i.e. in early $G_1$, cyclin $E_T$ expression was already maximally induced (~6-fold), remained constant at a lower level during $G_1$ and S, decreased further as the cells proceeded through $G_2$ and M and increased again in the subsequent early $G_1$ phase. These results clearly show that the pattern of cyclin $E_T$ expression differs from those of cyclin $E_L$ and $E_S$ during both the $G_0 \rightarrow S$ and $G_1 \rightarrow S$ progressions.

## Cyclin $E_T$ expression is repressed during terminal differentiation

In view of the unique expression pattern of cyclin $E_T$ during cell cycle entry and progression, we finally asked the question how is cyclin $E_T$ expression regulated in cells exiting from the cell cycle during terminal differentiation? As a model system we used the promyelocytic HL-60 cell line, which can be induced by either RA or TPA to differentiate into granulocyte-like or macrophage-like cells respectively (30,31). Unexpectedly, in both differentiated cell types cyclin $E_T$ expression was dramatically reduced (Fig. 8). To exclude an *in vitro* artefact, fresh granulocytes and monocytes from peripheral blood samples were also analysed. As shown in Figure 8, this analysis yielded a very similar result, i.e. a relatively strong repression of cyclin $E_T$. To investigate this finding in greater detail, cyclin $E_T$ expression was monitored at various time points after induction of differentiation by either RA (Fig. 9) or TPA (Fig. 10). Regardless of the inducing agent, cyclin $E_L$ expression remained relatively constant during the early time points and declined in the late stages of differentiation. In contrast, cyclin $E_T$ expression was initially induced, raising the possibility that progression through a certain cell cycle stage in $G_1$, where cyclin $E_T$ expression is induced, might be necessary to enter terminal differentiation. As shown in Figure 8, cyclin $E_T$ expression strongly decreased in the late stages of differentiation.

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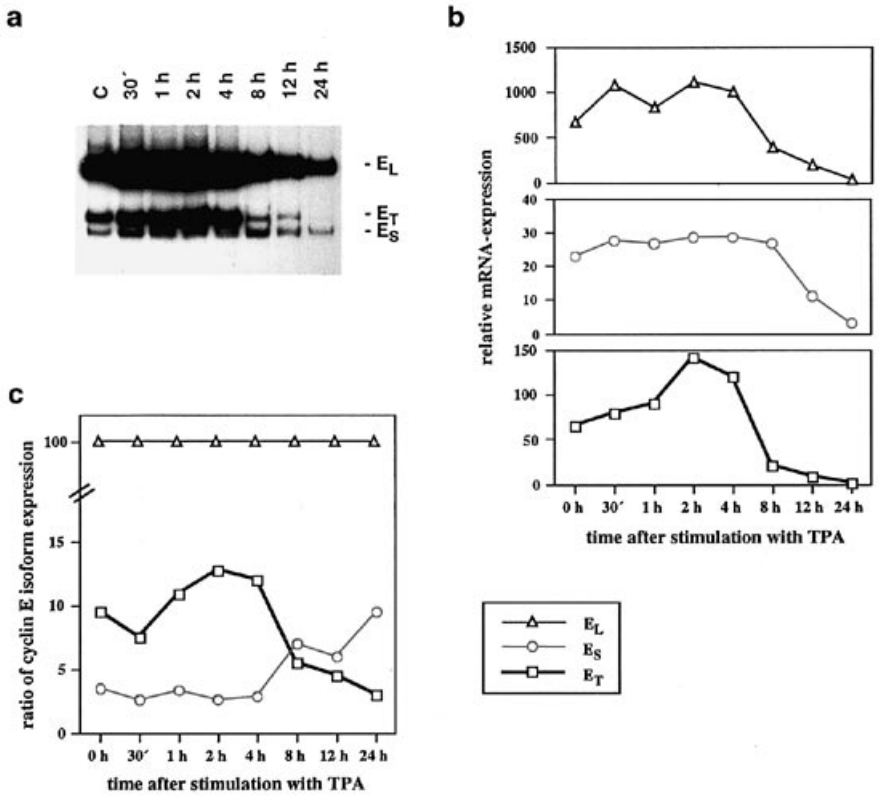


**Figure 10.** Expression of cyclin E splice variants in differentiating HL-60 cells after stimulation with TPA. **(a)** Human HL-60 cells were induced to differentiate into macrophage-like cells by stimulation with TPA. RNA was isolated at the indicated time points after stimulation and analysed via RT–PCR. C, normally proliferating HL-60 cells. **(b)** Quantitative evaluation of the results obtained in (a) and standardization for β-actin expression. The lowest expression level (cyclin $E_T$/24 h) was assigned the arbitrary value of 1. **(c)** Ratio of cyclin $E_T$ and $E_S$ expression relative to cyclin $E_L$. The expression levels of cyclin $E_T$ and $E_S$ are expressed as a percentage of the corresponding level of cyclin $E_L$ expression.

A quantitative evaluation of the data showed, however, that cyclin $E_T$ was specifically repressed in the macrophage-like cells (Fig. 10c), whilst the decrease in cyclin $E_T$ expression paralleled that of cyclin $E_L$ during differentiation of HL-60 cells into granulocyte-like cells (Fig. 9c). These results indicate that expression of cyclin $E_T$ during terminal differentiation is regulated in a lineage-specific fashion. Interestingly, cyclin $E_S$ was clearly induced in the late stages of both HL-60 cell differentiation pathways, showing that expression and/or splicing of cyclin $E_S$ also occurs in a specific and regulated manner, at least in certain instances. This observation is in apparent contradiction of the results of a previous study (34), which showed that expression of cyclin $E_S$ during terminal differentiation of HL-60 cells paralleled that of cyclin $E_L$. However, because of the very similar sizes of cyclin $E_S$ and $E_T$, the expression levels measured for cyclin $E_S$ presumably reflect the combined expression of both isoforms, thereby blurring the differential regulation. In summary, our results show that expression of cyclin $E_T$, and to some extent of cyclin $E_S$, is specifically regulated during terminal differentiation *in vitro* and *in vivo*.

## CONCLUSIONS

In this study we have identified a new alternatively spliced isoform of cyclin E, cyclin $E_T$. Expression of cyclin $E_T$ clearly differs from that of cyclin $E_L$, being induced early during cell cycle entry and progression and being strongly repressed during terminal differentiation of myeloid cells. Cyclin $E_T$ is unable to function as a $G_1$ cyclin in yeast, although it contains an intact cyclin box. These results show that the cyclin box, although required, is insufficient for the function of cyclin E. At present it is, however, only possible to speculate about the biological function of cyclin $E_T$. Cyclin E has been detected in several complexes with various other proteins, like p107, E2F, p21 and p27 (for reviews see 2,3,5–7) and cyclin $E_T$ may be able to bind to one or more of these proteins, thereby interfering with the formation or normal function of these complexes. Furthermore, the unique expression pattern of cyclin $E_T$ during cell cycle entry and progression strongly suggests a specific function for cyclin $E_T$ in these processes. In addition, the cell type-specific expression pattern of cyclin $E_T$ and cyclin $E_S$ during myeloid differentiation might indicate a role for cyclin E splice variants in terminal differentiation, similar to observations made with D-type cyclins (42). Apart from cyclin $E_T$, two further spliced isoforms of human cyclin E have been described (23,27). The present study, however, shows for the first time that alternative splicing of human cyclin E is not a constitutive process but is regulated during cell cycle progression and differentiation. Therefore, the alternative splicing of cell cycle regulatory molecules may represent an additional mechanism of cell cycle control in higher eukaryotes. The fact that the existence of an alternatively spliced variant has also been described recently for the cyclin E catalytic partner cdk2 (43) strongly supports this hypothesis.

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