Journal of Cell Science 107, 581-588 (1994)
Printed in Great Britain © The Company of Biologists Limited 1994
JCS2043

Alternative splicing of human cyclin E

Andreas Sewing*, Volker Rönicke*, Christiane Bürger, Martin Funk and Rolf Müller†

Institut für Molekularbiologie und Tumorforschung (IMT), Philipps-Universität Marburg, Emil-Mannkopff-Strasse 2, D-35037 Marburg, Germany

*The first two authors contributed equally to this work
†Author for correspondence

SUMMARY

Cyclin E is a regulatory subunit of the cdc2-related protein kinase cdk2, which is activated shortly before S-phase entry, thus defining it as a G_1 cyclin. We report here the existence of a 43 kDa splice variant of human cyclin E, termed cyclin Es, which lacks 49 amino acids within the cyclin box compared to the known 48 kDa cyclin E. Cyclin Es is expressed at approximately 1/10 of the level of full-length cyclin E in several cell lines analysed. The two cyclin E forms differ functionally in that cyclin E, but not cyclin Es, 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 *S. cerevisiae*. Cyclin Es is the first splice variant of a cell cycle regulatory protein to be described. Our findings also indicate that the cyclin box in cyclin E mediates the interaction with cdk2.

Key words: cell cycle, cyclin E, gene regulation, splicing

INTRODUCTION

The cyclins represent a major group of cell cycle regulatory proteins present in all eukaryotic cells. They were originally identified as proteins that accumulate during the cell cycle and are degraded in mitosis (for reviews see Hunt, 1989; Hunter and Pines, 1991; Lew and Reed, 1992; Lewin, 1990; Müller et al., 1993; Nigg, 1993; Sherr, 1993), thus controlling entry of the cell into M-phase. The mitotic cyclins function by modulating the activity of the serine-threonine protein kinase cdc2/CDC28. More recently, another group of cyclins was detected whose members do not show the pattern of expression and destruction typical of the mitotic cyclins. Some members of this group have been shown to play a pivotal role in the regulation of S-phase entry, most notably the CLN1, CLN2 and CLN3 genes in *S. cerevisiae* (for reviews see Herskowitz, 1988; Nasmyth et al., 1991; Müller et al., 1993). Mammalian cells contain, besides the mitotic A and B-type cyclins, another set of structurally related proteins (Koff et al., 1991; Lew et al., 1991; Matsushime et al., 1991; Xiong et al., 1991) which, like the CLN proteins in *S. cerevisiae*, seem to play a role in $G_1 \rightarrow S$ progression (for a recent review see Sherr, 1993). The best characterised cyclins of this category are the D-type cyclins and cyclin E. These human cyclins were identified by virtue of their ability to rescue *S. cerevisiae* cells lacking all 3 CLN gene products (triple CLN mutants) (Koff et al., 1991; Lew et al., 1991; Xiong et al., 1991). Murine D-type cyclins (previously called *cyl*) were identified independently by differential screening of a cDNA library from CSF-1-stimulated mouse macrophages (Matsushime et al., 1991), and as a putative proto-oncogene associated with chromosomal translocations, gene amplifications and proviral insertions in human and murine tumours (Lammie et al., 1991, 1992; Motokura et al., 1991; Rosenberg et al., 1991).

The D-type cyclins, D1, D2 and D3, associate with different proteins, such as cdk2, cdk4, cdk5, the retinoblastoma suppressor protein pRb, the DNA polymerase δ subunit PCNA and an unidentified protein termed p21 (Matsushime et al., 1992; Xiong et al., 1992; Dowdy et al., 1993; Ewen et al., 1993; Kato et al., 1993). The role of these diverse interactions in cell cycle regulation remains however largely elusive. It has been suggested that cdk4 is the natural catalytic subunit of cyclin D (Matsushime et al., 1992), since its kinase activity is not significantly activated by any of the other known cyclins expressed in a baculovirus system (Ewen et al., 1993; Kato et al., 1993). Complexes between D-type cyclins and the tumour suppressor gene product pRb, and cdk4/cyclin D-mediated pRb phosphorylation, have been detected in baculovirus-infected insect cells, although there is some controversy as to the function of individual members of the cyclin D family (Dowdy et al., 1993; Ewen et al., 1993). To date, however, cyclin D-associated kinase activity in mammalian cells has not been demonstrated. It has also been shown that D-type cyclins can rescue cells arrested in G_1 by the ectopic expression of pRb (Hinds et al., 1992; Dowdy et al., 1993; Ewen et al., 1993), but this function of cyclin D apparently does not require its physical interaction with pRb and may therefore be associated with a process located further downstream of pRb inactivation (Ewen et al., 1993). Recently, it has been shown that the ectopic expression of cyclin D1 leads to a shorter G_1-phase (Ewen et al., 1993) and, conversely, that the inhibition of cyclin D1 by microinjected antibodies or antisense RNA arrests cells in G_1 (Baldin et al., 1993; Ewen et al., 1993). From

these data it seems very likely that D-type cyclins fulfil a critical role in $G_1 \rightarrow S$ progression.

The picture appears somewhat clearer with respect to cyclin E, a regulatory subunit of cdk2. Cyclin E forms a complex with cdk2 and activates its serine-threonine activity shortly before S-phase entry (Dulic' et al., 1992; Koff et al., 1992). This leads to the hypothesis that cyclin E/cdk2 complexes might play a direct role in controlling the onset of DNA replication. In agreement with this conclusion is the observation that the inhibition of cdk2 causes a G_1 block (Tsai et al., 1993), while the ectopic expression of cyclin E leads to a shortening of G_1 (Ohtsubo and Roberts, 1993). In addition, cyclin E has been shown to release a pRb-induced G_1 block (Hinds et al., 1992), as described above for D-type cyclins. The situation is, however, more complex, since later in S-phase cyclin E is found not only in complexes with cdk2, but also with the pRb-related protein p107 and members of the E2F transcription factor family (Lees et al., 1992). Similar complexes are also seen with cyclin A (Mudryj et al., 1991; Bandara et al., 1991, 1992; Devoto et al., 1992; Ewen et al., 1992; Faha et al., 1992; Lees et al., 1992), which seems to have multiple functions in that it is required for both S-phase progression and entry into mitosis (Girard et al., 1991; Walker and Maller, 1991; Pagano et al., 1992).

In the present study, we have identified a new splice variant of human cyclin E, which differs from its known full-length counterpart by a 49 amino acid deletion in the cyclin box. This shorter cyclin E form is defective in cdk2 binding and activating cdk2 kinase activity, indicating that cdk2 binding is dependent on a functional cyclin box in cyclin E. In addition, cyclin Es is unable to rescue a triple CLN mutant of *S. cerevisiae* or to interfere with a cyclin E-mediated rescue.

MATERIALS AND METHODS

Cell culture

HL-60 cells were grown in RPMI-1640 supplemented with 5% fetal calf serum (FCS) plus 5% newborn calf serum. All other cell lines were cultured in Dulbecco-Vogt modified Eagle's minimum essential medium (DMEM) supplemented with 10% FCS and 0.5% glucose. Serum stimulation of quiescent WI-38 cells was carried out as described (Sewing et al., 1993).

Detection of RNA by polymerase chain reaction (RNA-PCR)

RNA-PCR was performed as previously described (Mumberg et al., 1991; Sewing et al., 1993). The following primers were used in this study:
cyclin E (Koff et al., 1991):
P54 5′-ATGGCTCGAGACACCATGAAGGAGGACGGC-3′ (–4-15)
P1194 5′-AACGGAATTCGGTGGTCACGCCATTTCCGG-3′ (1174-1195)
P387 5′-GACATACTTAAGGGATCAGC-3′ (387-406)
P742 5′-GGGGACTTAAACGCCACTTA-3′ (727-742)
p107 (Ewen et al., 1991):
5′-CGAGGATCCAGCAGTCATTACTCTTGTTGT-3′ (744-764)
5′-GATCTCGAGATTCGCCAAGTCGTATTTCAG-3′ (2440-2460)
cdk2 (Tsai et al., 1991):
5′-GGAGTGGATCCATGGAGAACTTCCAAAAG-3′ (170-189)
5′-TTGAGAATTCTATCAGAGTCGAAGATGGGG-3′ (278-298)

Southern blotting

Southern blotting of genomic DNA was carried out at described (Mumberg et al., 1991).

Expression of GST fusion proteins in *E. coli*

Human cyclin E, pRB, and p107 were expressed in bacteria using the GST-expression system (Pharmacia-LKB). cDNAs encoding the entire cyclin E open reading frame (*Xho*I/*Eco*RI fragment), amino acids 300-928 of pRB (*Bam*HI/*Eco*RI) and amino acids 248-821 of p107 (*Bam*HI/*Xho*I) were cloned into appropriate sites of pGEX-3XP, which had been generated by insertion of a new polylinker (GATCACGGATCCATGGTACCGCGGAGCTCGAGAAGCTTG) into the *Bam*HI/*Eco*RI sites of pGEX-3X (Pharmacia-LKB). Expression, induction, and purification were performed according to the instructions of the manufacturer.

Generation and purification of antibodies

Bacterially expressed cyclin E fusion protein was used for the production of antisera. Castor Rex rabbits were immunised with 250 μg of protein emulsified in ABM-S (Linaris, Germany) and boosted at three week intervals. Small scale affinity purification was performed according to Harlow and Lane (1988) using baculovirus-expressed recombinant cyclin E.

Generation of recombinant baculoviruses

Cyclin E, Es, A, and cdk2 cDNAs encoding the respective entire open reading frames were cloned into the baculovirus transfer vector pVL1392 (Pharmingen). For generation of recombinant viruses, linearized Baculogold DNA (Pharmingen) was cotransfected with the transfer vector into Sf9 cells using the Lipofection reagent (Gibco-BRL). Growth of cells and viral propagation were carried out as described (Summers and Smith, 1987).

Immunoprecipitation and immunoblotting

Immunoprecipitation of cyclin E and cdk2 protein complexes from metabolically labelled Sf9 cells was carried out as described (Sewing et al., 1993), except that the cells were labelled with 200 μCi of $[^{35}S]$methionine/ml of medium for 3 hours. Immunoblotting was performed as described (Sewing et al., 1993), with the exception that affinity-purified cyclin E antibodies were used.

In vitro protein kinase assay

Forty to forty-four hours post-infection, 2×10^6 infected Sf9 cells were harvested and sonicated (2× 5 seconds, 30 W) at 4°C in 200 μl of kinase buffer (50 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$, 1 mM DTT, 1 mM PMSF, 0.1% aprotinin, 20 mM NaF, 10 mM β-glycerophosphate) and lysates were cleared by centrifugation. For kinase reactions 4 μl lysate (15-20 μg protein) were incubated with either 10 μg histone H1, 5 μg GST-RB, or GST-p107 in 20 μl kinase buffer with 100 μM ATP and 10 μCi $[\gamma\text{-}^{32}P]$ATP (5000 Ci/mmol, Amersham) for 20 minutes at 30°C.

Yeast strains and techniques

The strain DL1 (*MAT*α; *ade1*; *his2*; *leu2-3*, *112*; *trp1-1a*; *ura3; cln1*::*TRP1*; *cln2*; *cln3*; *leu2*::*GAL1*::*CLN2*), was kindly provided by Steve Reed. DL1 was grown on YPG (1% yeast extract, 2% Bacto-Peptone, 4% galactose) and on selective medium SD or SG (Ausubel, 1987) with or without 1 mM methionine. Cyclin E and Es expression vectors are based on the plasmids pRS416 (Sikorski and Hieter, 1989), pRS425 and pRS426 (Christianson et al., 1992). The MET25 (Thomas et al., 1989) and ADH (Bennetzen and Hall, 1982) promoters were inserted as PCR-generated *Sac*I/*Xba*I-fragments and the CYC1-terminator (Osborne and Guarente, 1989) as a PCR-generated *Xho*I/*Kpn*I-fragment. Transformations were carried out according to Gietz et al. (1992). Proteins were extracted using the glass bead method (Ausubel, 1987) and lysis buffer (50 mM Tris, pH7.5, 150 mM $MgCl_2$, 1% NP-40, 40 mg/l aprotinin, 0.1 mM sodium orthovanadate, 1 mM DTT, 1 mM PMSF and 60 mM β-glycerophosphate).

RESULTS AND DISCUSSION

Detection of two mRNAs encoding different forms of human cyclin E: evidence for alternative splicing

In the course of analysing the expression of the cyclin E gene in different cell lines we observed a second minor RNA species that was slightly smaller than the major RNA representing the previously described cyclin E (Koff et al., 1991). This shorter cyclin E RNA, termed cyclin Es, was detected by PCR analysis of cDNA from 6 different cell lines, i.e. the small cell lung carcinoma cell line NCI-H69, HepG2 hepatoma cells, the lung adenocarcinoma cell line A549, the human diploid fibroblast line WI-38, promyelocytic HL-60 cells and the cervical carcinoma cell line HeLa (Fig. 1). In order to verify this result, the experiment was repeated using a different PCR primer pair with cDNA synthesised from HeLa cell RNA. In both cases, the major upper bands corresponded to the expected sizes of 688 bp and 355 bp, respectively, while the minor lower bands represented fragments that were approximately 150 nucleotides shorter. We also analysed the expression of cyclin E and Es mRNA in serum-stimulated WI-38 cells. As shown in Fig. 1c, both cyclin E forms were coordinately expressed over the investigated period of 27 hours post-stimulation, indicating that at least under these conditions the alternative splicing of the transcript is not regulated.

A closer inspection of the nucleotide sequence between positions 387 and 742 revealed the presence of potential splice donor (SD; AG/GTGAGA) and splice acceptor (SA; CAG) sites (see Fig. 2b), which might lead to the expression of an alternative mRNA with an in-frame deletion of 147 nucleotides compared to full-length cyclin E. To test this hypothesis, we isolated the smaller amplified fragment by agarose gel electrophoresis and cloned it into pBluescript KS. The DNA sequence of the insert was determined and compared to the corresponding region of cyclin E. The sequence of the relevant region around the splice junction is shown in Fig. 2a and b. It is obvious that the sequence of cyclin Es is identical to that of cyclin E until the position of the SA site is reached and merges with the cyclin E sequence immediately 3′ of the SA site. This strongly suggests that cyclin Es is indeed an alternative splice product of the cyclin E transcript. As shown in Fig. 2c, the putative product of cyclin Es lacks 49 amino acids within the cyclin box, which includes a region that in cyclin A has been shown to be required for cdc2 and cdk2 binding (Kobayashi et al., 1992; Lees and Harlow, 1993).

The two cyclin E mRNA forms are derived from a single gene

In order to verify that the two mRNA forms are indeed generated by alternative splicing of a single primary transcript rather than by the transcription of two related genes we performed Southern blot analyses of human genomic DNA (Fig. 3). DNA from lymphocytes and HeLa cells was digested with either *Bam*HI or *Eco*RI, and the blot was hybridised to a cDNA probe covering the cyclin E coding region. It is known that the cyclin E cDNA contains one *Bam*HI and no *Eco*RI site (Koff et al., 1991). The observed number of hybridising fragments (2 for *Bam*HI; 1 for *Eco*RI) shows that the cyclin E gene does not contain additional *Bam*HI or *Eco*RI sites and indicates that all fragments were derived from a single gene. This result therefore supports the conclusion that cyclin Es is generated by alternative splicing.

Identification of cyclin Es protein

In an attempt to identify the cyclin Es gene product and to detect potential functional differences between cyclin E and cyclin Es we expressed both cDNAs in insect Sf9 cells by

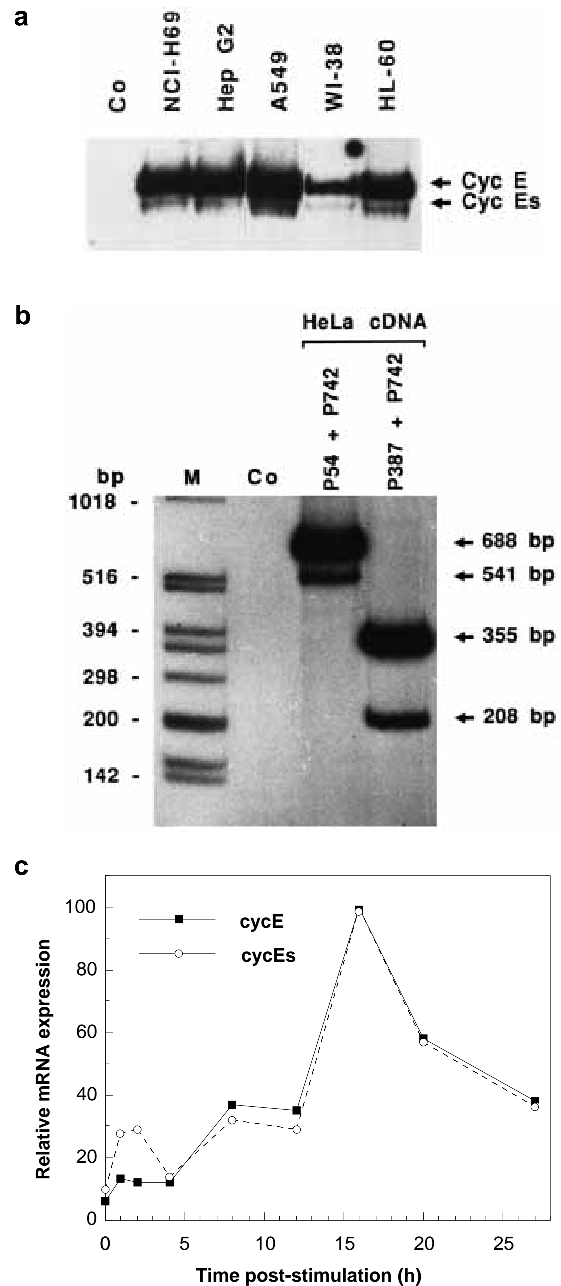

Fig. 1. (a,b) Detection of two PCR products in cDNA from different cell lines using cyclin E-specific primer pairs (P54/P742 in (a) and (b); P387/P742 in (b)). M, size markers; Co, control without cDNA input. (c) Expression of cyclin E and Es in serum-stimulated WI-38 human fibroblasts. Results obtained by RNA-PCR were quantitatively evaluated by β-radiation scanning (Molecular Dynamics PhosphorImager) and normalised for L7 expression as described (Sewing et al., 1993).

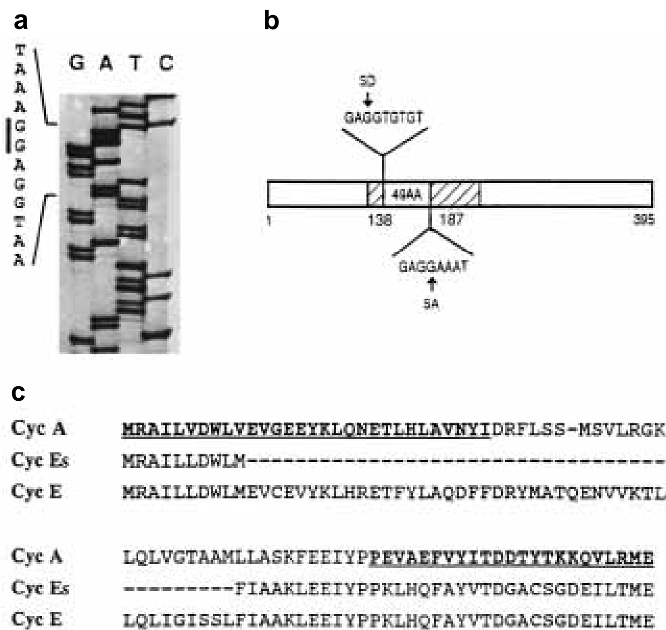

Fig. 2. Identification of cyclin Es as a splice variant. (a) Nucleotide sequence of cyclin Es around the site of deviation from cyclin E. The splice junction is marked by a vertical line. (b) Schematic representation of cyclin E protein (Koff et al., 1991) indicating the deletion in cyclin Es (49AA) and the splice donor (SD) and splice acceptor (SA) sites in the corresponding nucleotide sequence. The cyclin box is shown as a striped box. Numbers refer to amino acid positions. (c) Comparison of the cyclin box regions in cyclin A, cyclin E and cyclin Es. Sequences in cyclin A required for interaction with cdc2 (Lees and Harlow, 1993) are marked by underlining.

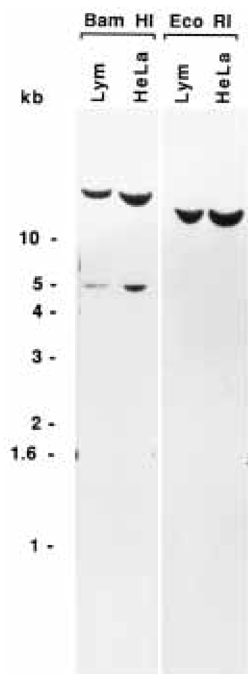

Fig. 3. Southern blot analysis of DNA from human lymphocytes and HeLa cells after digestion with either *Bam*HI or *Eco*RI, using a probe covering the cyclin E coding region. The numbers on the left show the positions of the size marker bands.

means of a baculovirus expression vector. In addition, antibodies were raised in rabbits against a GST-cyclin E fusion protein expressed in *E. coli*. As shown in Fig. 4a, these antibodies (αK49) immunoprecipitated cyclin E protein synthesised by in vitro transcription/translation, and this binding was inhibited by competitor GST-cyclin E protein, confirming the specificity of the antibodies.

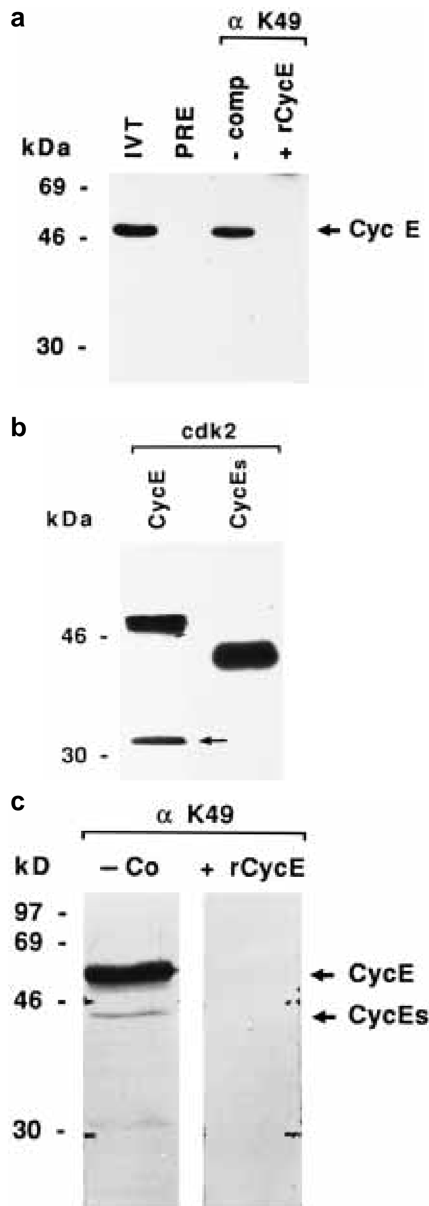

Fig. 4. (a) Specificity of the α-cyclin E antibody αK49. IVT, cyclin E protein generated by in vitro transcription/ translation; PRE, cyclin E immunoprecipitated with preimmune serum; αK49, cyclin E immunoprecipitated with the αK49 antiserum in the presence (+rCyc E) or absence (–comp) of competing recombinant GST-cyclin E protein. (b) Co-immunoprecipitation of cdk2 (arrow) with cyclin E but not cyclin Es from metabolically ([^{35}S]methionine)-labelled insect Sf9 cells co-infected with baculoviruses expressing cyclin E, cyclin Es or cdk2, using the αK49 antiserum. (c) Detection of two cyclin E proteins of approximately 48 and 43 kDa in HeLa cells by immunoblotting using affinity-purified αK49 antibodies. The specificity of the immunodetection was shown by competition with recombinant GST-cyclin E (+rCycE, right lane). –Co, no competitor.

Sf9 cells infected with recombinant cyclin E and cyclin Es baculoviruses were metabolically labelled with [^{35}S]methionine and cell extracts were immunoprecipitated using αK49 antibodies. This experiment showed that both cyclin E and cyclin Es were expressed in the infected insect cells, giving rise to proteins of approximately 48 kDa and 43 kDa, respectively, corresponding to the expected sizes (Fig. 4b). Proteins of very similar size were identified by immunoblotting in HeLa cells using affinity-purified αK49 antibodies (Fig. 4c). Both bands disappeared when the antibodies were incubated with recombinant GST-cyclin E, indicating the specificity of the immunodetection. The two cyclin Es proteins were expressed at a similar ratio as the corresponding mRNAs, i.e. cyclin Es protein was found at about 1/10 to 1/20 of the level of cyclin E (compare Figs 1 and 4c). These findings show that both

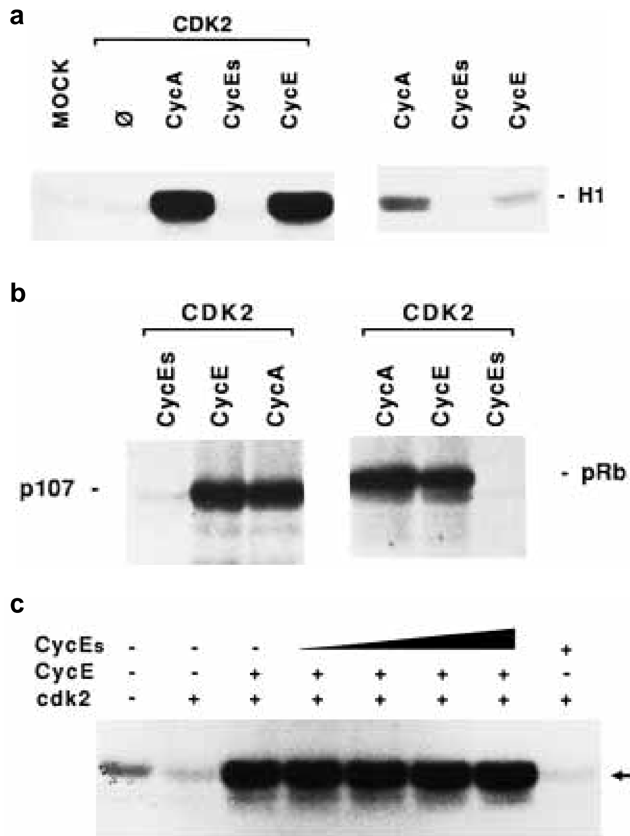

Fig. 5. Activation of cdk2 kinase activity by cyclin A and cyclin E, but not by cyclin Es in immune complexes precipitated from doubly infected insect cells (as in Fig. 4), using histone H1 (a), or fragments of p107 or pRb (b) as the substrate. The latter two proteins were GST fusion products expressed in *E. coli*. (c) cdk2/cyclin E-dependent phosphorylation of histone H1 in the presence of increasing amounts of cyclin Es (from left to right 1-, 2-, 5- and 10-fold molar excess of cyclin Es). Extracts from Sf9 cells infected with either cdk2 or cyclin Es-expressing baculoviruses were mixed in vitro, preincubated for 30 minutes prior to the addition of cyclin E extract and incubation with the substrate after another 30 minutes. The arrow points to the histone H1 band.

cyclin E mRNA forms are translated into readily detectable proteins in vivo, and strongly suggest that both cyclin E and cyclin Es are expressed as endogenous proteins in HeLa cells.

Cyclin E, but not cyclin Es, binds to and activates cdk2

We next analysed the interaction of cyclin E and cyclin Es with cdk2. For this purpose, we generated a recombinant baculovirus expressing cdk2 to be able to perform double infections with this virus and either of the cyclin-expressing viruses. The data in Fig. 4b show that cdk2 coprecipitated with cyclin E, as expected (Dulic' et al., 1992; Koff et al., 1992), but not with cyclin Es. In addition, we measured the protein serine-threonine kinase activity in Sf9 cells coinfected with cdk2 and either cyclin A (as a control), cyclin E or cyclin Es, or with either of the three cyclins alone. The data displayed in Fig. 5a clearly show that cyclin A-, cyclin E- and cyclin Es-expressing cells contained only marginal histone H1 kinase activity, if any, and that the coexpression of either cyclin A or cyclin E led to a dramatic increase in activity, which is in agreement with published data (Dulic' et al., 1992; Giordano et al., 1991; Koff et al., 1992; Tsai et al., 1991). The same experiments were performed with pRb and p107 as the substrate with very similar results (Fig. 5b). These data also demonstrate that, in contrast to cyclin E, cyclin Es is unable to interact with cdk2 and to activate its protein kinase activity.

We also analysed whether cyclin Es might be able to interfere with the kinase activity of cdk2/cyclin E complexes. For this purpose, cdk2/cyclin E-dependent phosphorylation of histone H1 was determined in the presence of increasing amounts of cyclin Es (1- to 10-fold molar excess of cyclin Es; see Fig. 5c). Extracts from Sf9 cells infected with either cdk2- or cyclin Es-expressing baculoviruses were mixed in vitro, preincubated for 30 minutes, then incubated with cyclin E extract for another 30 minutes and finally incubated with histone H1 as the substrate. The results shown in Fig. 5c clearly indicated that even in the presence of a 10-fold molar excess of cyclin Es the cdk2/cyclin E-directed kinase activity was not affected.

Cyclin Es is unable to rescue a triple CLN mutant of *S. cerevisiae*

Human cyclin E was originally discovered by its ability to rescue a triple CLN mutant of *S. cerevisiae* (Koff et al., 1991; Lew et al., 1991). We therefore decided to investigate whether cyclin Es is also defective in this function, as suggested by its inability to activate cdk2, and, if so, whether it might antagonise the function of cyclin E. The later question was of particular interest, since the cyclin E-mediated rescue seems to involve interactions outside the cyclin box (our unpublished observations), raising the possibility that cyclin Es might squelch functionally crucial proteins. For this purpose, cyclin D1 (for comparison), cyclin E and cyclin Es were expressed under the control of the methionine repressible MET25 promoter in the *S. cerevisiae* strain DL1 whose CLN genes were changed by homologous recombination in such a way that CLN1 and 3 function was deleted and CLN2 was under the control of the GAL promoter (Lew et al., 1991). The MET25 promoter is repressed approximately 100-fold in the presence of methionine (D. Mumberg and M. Funk, unpublished observation).

Fig. 6a shows that in the presence of galactose, that is, in the presence of CLN2, all clones were able to grow, irrespective of the presence of cyclin D1, cyclin E or cyclin Es (Gal 1mM Met). In contrast, in the absence of CLN2 (i.e. in the presence of glucose), cells not expressing exogenous cyclin or cyclin Es did not grow, while cyclin D1 and cyclin E were able to rescue the triple CLN mutant (Fig. 6a, bottom panel). In the case of cyclin E cell growth was detectable only at a low level of expression, that is, in the presence of the repressing methionine (Glc 1mM Met), while the level of cyclin D1 expression did not seem very crucial. This observation confirms the described toxic effect of cyclin E when expressed at high levels (Lew et al., 1991).

In another set of experiments cyclin Es was co-expressed with cyclin E in order to detect potential antagonistic effects. In this case, cyclin E was expressed under the control of the ADH promoter on a centromer plasmid while cyclin Es was driven by the MET25 promoter on a 2μ plasmid. In the absence of methionine, the MET25 expression vector gives rise to

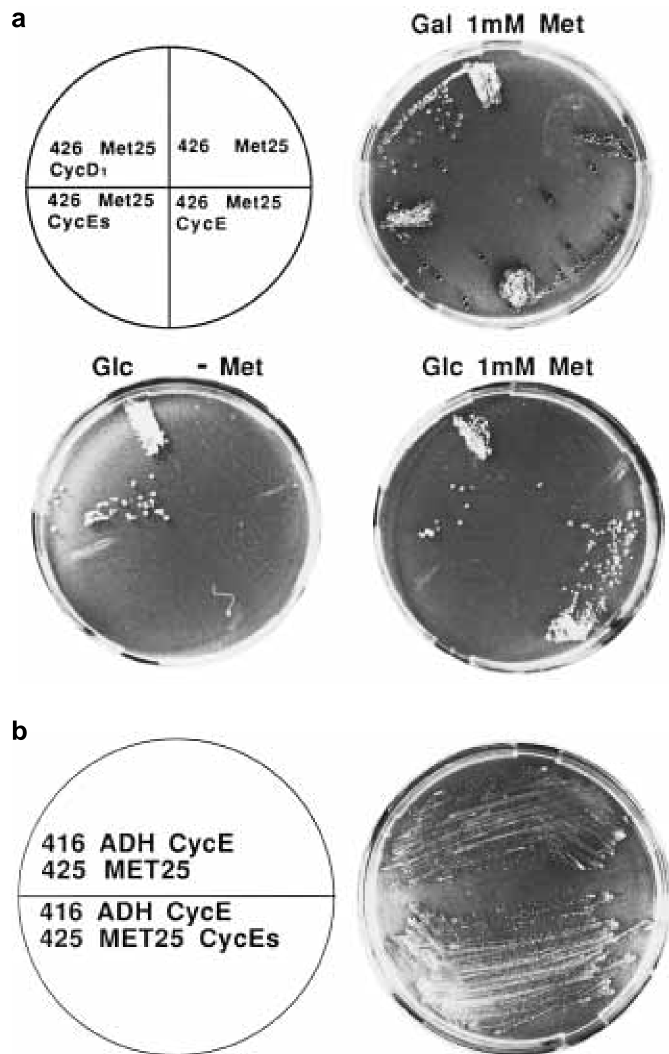

Fig. 6. (a) Rescue of triple CLN *S. cerevisiae* mutants by cyclin D1 and cyclin E, but not by cyclin Es. The expression vectors indicated in the top left panel were transformed into the strain DL1 and growth was monitored under different conditions. Gal 1mM Met, cells grown in galactose plus 1mM methionine; Glc-Met, cells grown in galactose in the absence of methionine; Glc 1mM Met, cells grown in glucose plus 1 mM methionine. (b) cyclin Es does not interfere with the ability of cyclin E to rescue a triple CLN mutant. The plasmids indicated in the left panel were cotransferred into GAL-CLN2 cells (grown in glucose) and growth was monitored in the absence of methionine. The strength of the promoters as measured with lacZ fusion genes is as follows (arbitrary units): ADH, 1; MET25 + 1 mM methionine, 2; MET25 in the absence of methionine: 200 (see text for details).

approximately 200-fold higher expression levels than the ADH vector, as determined by comparing the activity of the respective promoter-lacZ constructs (D. Mumberg and M. Funk, unpublished observation). This experimental setup was chosen to allow a strong overexpression of cyclin Es relative to cyclin E. However, as shown in Fig. 6b, even under these conditions cyclin Es did not affect the ability of cyclin E to rescue the triple CLN mutant cells.

In order to verify that both cyclin E forms were indeed expressed in the yeast cells, we performed immunoblot analyses of cells transformed with either MET25-cyclin E or MET25-cyclin Es constructs. Cells expressing cyclin E or cyclin Es were pre-grown on selective minimal medium supplemented with 1 mM methionine and shifted to methionine-free medium for 6 hours in order to achieve a comparably high expression of both constructs. As shown in Fig. 7, both proteins were clearly detectable using affinity-purified αK49 antibodies (see above and Fig. 4a), cyclin Es apparently being slightly stronger expressed than cyclin E. The faster migrating bands seen in Fig. 7 most likely represent degradation products of cyclin E and cyclin Es, respectively. The results of the immunoblot analysis, taken together with the data presented above, strongly suggest that cyclin Es is neither able to substitute for a CLN function in *S. cerevisiae* nor to interfere with the rescue by cyclin E.

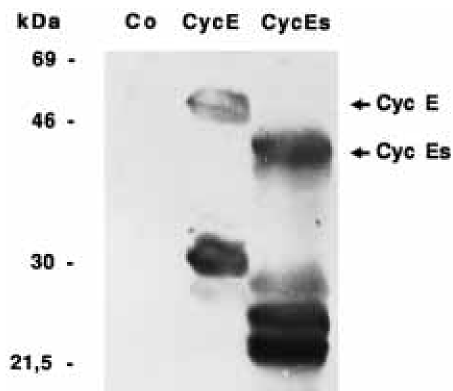

Fig. 7. Detection of human cyclin E and cyclin Es in transformed *S. cerevisiae* cells by immunoblotting using affinity-purified αK49 antibodies. The lower bands presumably represent degradation products of cyclin E and cyclin Es.

Conclusions

In this study, we have shown that a number of different human cell lines expresses a novel cyclin E mRNA generated by alternative splicing. Translation of the cyclin Es mRNA gives rise to a protein that differs from the known cyclin E gene product by an internal deletion of 49 amino acids within the cyclin box. This structural difference leads to a loss of cdk2 binding and activation, indicating that the cyclin box in cyclin E mediates the interaction with cdk2 and perhaps other related proteins. This conclusion is in agreement with the recently reported observation that parts of the cyclin box in cyclin A are required for the interaction with its catalytic subunits cdc2 and cdk2 (Kobayashi et al., 1992; Lees and Harlow, 1993). As shown in Fig. 2c, the N-terminal 20 amino acids of the region removed by alternative splicing overlaps with a homologous region in cyclin A shown to be indispensable for cdc2 binding (Lees and Harlow, 1993).

We also analysed the potential of cyclin Es to rescue a triple CLN mutant of *S. cerevisiae* and to interfere with the ability of cyclin E to do so. The latter experiment was of particular interest, since it provided the opportunity to detect a potential antagonistic function, but even a vast overexpression of cyclin Es did not show any detectable effect. In agreement with this result, we were also unable to detect an antagonistic function

of cyclin Es in an in vitro kinase assay using histone H1 as the substrate, at least within the range of 1- to 10-molar excess of cyclin Es.

These results, however, do not necessarily mean that cyclin Es has no biological function. In the case of D-type cyclins, for instance, it was shown that a N-terminally located domain outside the cyclin box mediates binding to the tumour suppressor gene product pRb (Dowdy et al., 1993), indicating that domains distinct from the cyclin box are involved in the interaction with other proteins. Unfortunately, functional domains other than the cyclin box, shown in the present study to mediate the interaction with cdk2, are not known for cyclin E. The search for a function for cyclin Es therefore has to await the results of more detailed structure-function analyses. It will be interesting to see whether such a putative function might be associated with the regulation of cyclin E activity or is otherwise related to the control of cell cycle progression.

We are grateful to Dr F. C. Lucibello and D. Mumberg for critical reading of the manuscript. This work was supported by the Deutsche Forschungsgemeinschaft (Mu601/5-2, Mu601/5-3 and Mu601/7-1) and the Dr Mildred Scheel-Stiftung für Krebsforschung. A.S. was the recipient of a fellowship from the Graduiertenkolleg ‘Zell- und Tumorbiologie’ at the Philipps-Universität Marburg.

REFERENCES

Ausubel I. and Frederick M. (1987). *Current Protocols in Molecular Biology*. Whiley & Sons, New York.

Baldin, V., Lukas, J., Marcote, M. J., Pagano, M. and Draetta, G. (1993). Cyclin D1 is a nuclear protein required for cell cycle progression in G_1. *Genes Dev.* **7**, 812-821.

Bandara, L. R., Adamczewski, J. P., Hunt, T. and La Thangue, N. B. (1991). Cyclin A and the retinoblastoma gene product complex with a common transcription factor. *Nature* **352**, 249-251.

Bandara, R. L., Adamczewski, J. P., Zamanian, M., Poon, R. Y. C., Hunt, T. and La Thangue, N. B. (1992). Cyclin A recruits $p33^{cdk2}$ to the cellular transcription factor DRTF 1. *J. Cell. Sci. Suppl. 16*, 77-85.

Bennetzen, J. and Hall, B. D. (1982). The primary structure of the Saccharomyces cerevisiae gene for alcohol dehydrogenase I. *J. Biol. Chem.* **257**, 3018-3025.

Christianson, T. W., Sikorski, R. S., Dante, M., Shero, J. H. and Hieter, P. (1992). Multifunctional yeast high-copy-number shuttle vectors. *Gene* **110**, 119-122.

Devoto, S. H., Mudryj, M., Pines, J., Hunter, T. and Nevins, J. R. (1992). A cyclin A-protein kinase complex possesses sequence-specific DNA binding activity: $p33^{cdk2}$ is a component of the E2F-cyclin A complex. *Cell* **68**, 167-176.

Dowdy, S. F., Hinds, P. W., Louie, K., Reed, S. I., Arnold, A. and Weinberg, R. A. (1993). Physical interaction of the retinoblastoma protein with human D cyclins. *Cells* **73**, 499-511.

Dulic', V., Lees, E., Reed, S. I. (1992). Association of human cyclin E with a periodic G_1-S phase protein kinase. *Science* **257**, 1958-1961.

Ewen, M. E., Xing, Y., Lawrence, J. B. and Livingston, D. M. (1991). Molecular cloning, chromosomal mapping, and expression of the cDNA for p107, a retinoblastoma gene product-related protein. *Cell* **66**, 1155-1164.

Ewen, M. E., Faha, B., Harlow, E. and Livingston, D. M. (1992). Interaction of p107 with cyclin A independent of complex formation with viral oncoproteins. *Science* **255**, 85-87.

Ewen, M. E., Sluss, H. K., Sherr, C. J., Matsushima, H., Kato, J. and Livingston, D. M. (1993). Functional interactions of the retinoblastoma protein with mammalian D-type cyclins. *Cell* **73**, 487-497.

Faha, B., Ewen, M. E., Tsai, L.-H., Livingston, D. M. and Harlow, E. (1992). Interaction between human cyclin A and adenovirus E1A-associated p107 protein. *Science* **255**, 87-90.

Gietz, D., Jean, A. S., Woods, R. A. and Schistl, R. H. (1992). Improved method for high efficiency transformation of intact yeast cells. *Nucl. Acids Res.* **20,** 1425.

Giordano, A., Lee, J. H., Scheppler, J. A., Herrmann, C., Harlow, E., Deuschle, U., Beach, D. and Franza Jr, B. R. (1991). Cell cycle regulation of histone H1 kinase activity associated with the adenoviral protein E1A. *Science* **253**.

Girard, F., Strausfeld, U., Fernandez, A. and Lamb, N. J. C. (1991). Cyclin A is required for the onset of DNA replication in mammalian fibroblasts. *Cell* **67**, 1169-1179.

Harlow, E. and Lane, D. (1988). *Antibodies: A Laboratory Manual*. Cold Spring Harbor, New York: Cold Spring Harbor Laboratory Press.

Herskowitz, I. (1988). Life cycle of the budding yeast *Saccharomyces cerevisiae*. *Microbiol. Rev.* **52**, 536.

Hinds, P. W., Mittnacht, S., Dulic, V., Arnold, A., Reed, S. I. and Weinberg, R. A. (1992). Regulation of retinoblastoma protein functions by ectopic expression of human cyclins. *Cell* **70**, 993-1006.

Hunt, T. (1989). Maturation promoting factor, cyclin and the control of M-phase. *Curr. Opin. Cell Biol.* **1**, 268.

Hunter, T. and Pines, J. (1991). Cyclins and cancer. *Cell* **66**, 1071-1074.

Kato, J., Matsushime, H., Hiebert, S. W., Ewen, M. E. and Sherr, C. J. (1993). Direct binding of cyclin D to the retinoblastoma gene product (pRb) and pRb phosphorylation by the cyclin D-dependent kinase CDK4. *Genes Dev.* **7**, 331-342.

Kobayashi, H., Stewart, E., Poon, R., Adamczewski, J. P., Gannon, J. and Hunt, T. (1992). Identification of the domains in cyclin A required for binding to, and activation of, $p34^{cdc2}$ and $p32^{cdk2}$ protein kinase subunits. *Mol. Biol. Cell* **3**, 1279-1294.

Koff, A., Cross, F., Fisher, A., Schumacher, J., Leguellec, K., Phiippe, M. and Roberts, J. M. (1991). Human cyclin E, a new cyclin that interacts with two members of the CDC2 gene family. *Cell* **66**, 1217-1228.

Koff, A., Giordano, A., Desai, D., Yamashita, K., Harper, J., W., Elledge, S., Nishimoto, T., Morgan, D. O. and Franza, R. J. M. (1992). Formation and activation of a cyclin E-cdk2 complex during the G_1 phase of the human cell cycle. *Science* **257**, 1689-1694.

Lammie, G. A., Fantl, V., Smith, R., Schuuring, E., Brookes, S., Michalides, R., Dickson, C., Arnold, A. and Peters, G. (1991). D11S287, a putative oncogene on chromosome 11q13, is amplified and expressed in squamous cell and mammary carcinomas and linked to bcl-1. *Oncogene* **6**, 439-444.

Lammie, G. A., Smith, R., Silver, J., Brookes, S., Dickson, C. and Peters, G. (1992). Proviral insertions near cyclin D1 in mouse lymphomas: a parallel for BCL1 translocation in human B-cell neoplasms. *Oncogene* **7**, 2381-2387.

Lees, E., Faha, B., Dulic', V., Reed, S. I. and Harlow, E. (1992). Cyclin E/edk2 and cyclin A/edk2 kinases associate with p107 and E2F in a temporally distinct manner. *Genes Dev.* **6**, 1874-1885.

Lees, E. M. and Harlow, E. (1993). Sequences within the conserved cyclin box of human cyclin A are sufficient for binding to and activation of cdc2 kinase. *Mol. Cell. Biol.* **13**, 1194-1201.

Lew, D. J., Dulic', V. and Reed, S. I. (1991). Isolation of three novel human cyclins by rescue of G_1 cyclin (Cln) function in yeast. *Cell* **66**, 1197-1206.

Lew, D. J. and Reed, S. I. (1992). A proliferation of cyclins. *Trends Cell Biol.* **2**, 77-80.

Lewin, B. (1990). Driving the cell cycle: M phase kinase, its partners, and substrates. *Cell* **61**, 743-752.

Matsushime, H., Roussel, M. F., Ashmun, R. A. and Sherr, C. J. (1991). Colony-stimulating factor 1 regulates novel cyclins during the G_1 phase of the cell cycle. *Cell* **65**, 701-713.

Matsushime, H., Ewen, M. E., Strom, D. K., Kato, J.-Y., Hanks, S. K., Roussel, M. F. and Sherr, C. J. (1992). Identification and properties of an atypical catalytic subunit ($p34^{PSK-J3}$/cdk4) for mammalian D-type G_1 cyclins. *Cell* **71**, 323-334.

Motokura, T., Bloom, T., Goo Kim, H., Jüppner, H., Ruderman, J. V., Kronenberg, H. M. and Arnold, A. (1991). A novel cyclin encoded by a bcl1-linked candidate oncogene. *Nature* **350**, 512-515.

Mudryj, M., Devoto, S. H., Hiebert, S. W., Hunter, T., Pines, J. and Nevins, J. R. (1991). Cell cycle regulation of the E2F transcription factor involves an interaction with cyclin A. *Cell* **65**, 1243-1253.

Müller, R., Mumberg, D. and Lucibello, F. C. (1993). Signals and genes in cell cycle progression. *Biochim. Biophys. Acta* (in press).

Mumberg, D., Lucibello, F. C., Schuermann, M. and Müller, R. (1991). Alternative splicing of fosB transcripts results in differentially expressed mRNAs encoding functionally antagonistic proteins. *Genes Dev.* **5**, 1212-1223.

Nasmyth, K., Dirick, L., Surana, U., Amon, A. and Cvrckova, F. (1991).

Some facts and thoughts on cell cycle control in yeast. In *The Cell Cycle* (ed. D. Beach, B. Stillman and J. D. Watson), pp. 9-20. Cold Spring Harbor, New York: Cold Spring Harbor Laboratory Press.

Nigg, E. A. (1993). Cellular substrates of $p34^{cdc2}$ and its companion cyclin-dependent kinases. *Trends Cell Biol.* **3**, 296-301.

Ohtsubo, M. and Roberts, J. M. (1993). Cyclin-dependent regulation of G_1 in mammalian fibroblasts. *Science* **259**, 1908-1912.

Osborne, B. I. and Guarente, L. (1989). Mutational analysis of a yeast transcriptional terminator. *Proc. Nat. Acad. Sci. USA* **86**, 4097-4101.

Pagano, M., Pepperkok, R., Verde, F., Ansorge, W. and Draetta, G. (1992). Cyclin A is required at two points in the human cell cycle. *EMBO J.* **11**, 961-971.

Rosenberg, C., Kim, H. G., Shows, T. B., Kronenberg, H. M. and Arnold, A. (1991). Rearrangement and overexpression of D11S287E, a candidate oncogene on chromosome11q13 in benign parathyroid tumors. *Oncogene* **6**, 449.

Sewing, A., Bürger, C., Brüsselbach, S., Schalk, C., Lucibello, F. C. and Müller, R. (1993). Human cyclin D1 encodes a labile nuclear protein whose synthesis is directly induced by growth factors and suppressed by cyclic AMP. *J. Cell Sci.* **104**, 545-555.

Sherr, C. J. (1993). Mammalian G_1 cyclins. *Cell* **73**, 1059-1065.

Sikorski, R. and Hieter, P. (1989). A system of shuttle vectors and yeast host strains designed for efficient manipulation of DNA in *Saccharomyces cerevisiae*. *Genetics* **122**, 19.

Summers, M. D. and Smith, G. E. (1987). *A Manual of Methods for Baculo Vectors and Insect Culture Procedures*. Texas Agricultural Experiment Bulletin no. 1555, College Station, TX.

Thomas, D., Cherest, H. and Surdin-Kerjan, Y. (1989). Elements involved in S-adenosylmethionine-mediated regulation of the *Saccharomyces cerevisiae* MET25 gene. *Mol. Cell. Biol.* **9**, 3292-3298.

Tsai, L.-H., Harlow, E. and Meyerson, M. (1991). Isolation of the human cdk2 gene that encodes the cyclin A- and adenovirus E1A-associated p33 kinase. *Nature* **353**, 174-177.

Tsai, L.-H., Lees, E., Faha, B., Harlow, E. and Riabowol, K. (1993). The cdk2 kinase is required for the G_1-to-S transition in mammalian cells. *Oncogene* **8**, 1593-1602.

Walker, D. H. and Maller, J. L. (1991). Role for cyclin A in the dependence of mitosis on completion of DNA replication. *Nature* **354**, 314-317.

Xiong, Y., Connolly, T., Futcher, B. and Beach, D. (1991). Human D-type cyclin. *Cell* **665**, 691-699.

Xiong, Y., Zhang, H. and Beach, D. (1992). D-type cyclins associate with multiple protein kinases and the DNA replication and repair factor PCNA. *Cell* **71**, 505-514.

(Received 10 September 1993 - Accepted 1 October 1993)