MOLECULAR AND CELLULAR BIOLOGY, July 1996, p. 3401–3409 Vol. 16, No. 7
0270-7306/96/$04.00+0

Cell Cycle Regulation of the Murine Cyclin E Gene Depends on an E2F Binding Site in the Promoter

JÜRGEN BOTZ,[1] KARIN ZERFASS-THOME,[1] DIMITRY SPITKOVSKY,[1] HAJO DELIUS,[1] BEATE VOGT,[1] MARTIN EILERS,[2] ARTEMIS HATZIGEORGIOU,[3] AND PIDDER JANSEN-DÜRR[1]*

Forschungsschwerpunkt Angewandte Tumorvirologie[1] and Forschungsschwerpunkt Bioinformatik,[3] Deutsches Krebsforschungszentrum, and Zentrum für Molekulare Biologie der Universität Heidelberg,[2] D-69120 Heidelberg, Germany

Received 27 December 1995/Returned for modification 10 February 1996/Accepted 12 April 1996

Cyclin E controls progression through the G_1 phase of the cell cycle in mammalian fibroblasts and potentially in many other cell types. Cyclin E is a rate-limiting activator of cdk2 kinase in late G_1. The abundance of cyclin E is controlled by phase-specific fluctuations in the mRNA level; in mammalian fibroblasts, mRNA is not detected under conditions of serum starvation and is accumulated upon serum stimulation, with expression starting in mid-G_1. Here, we report the cloning of the murine cyclin E promoter. We isolated a 3.8-kb genomic fragment that contains several transcriptional start sites and confers cell cycle regulation on a luciferase reporter gene. This fragment also supports transcriptional activation by adenovirus E1A, a known upstream regulator of cyclin E gene expression. An E2F binding site which is required for G_1-specific activation of the cyclin E promoter in synchronized NIH 3T3 cells was identified in this fragment.

Regulation of cell cycle progression in mammalian cells depends on the sequential activation of a series of cyclin-dependent kinases. The catalytic activity of a given cyclin-dependent kinase subunit depends on its correct posttranslational modification and on its association with the appropriate cyclin, which acts as a regulatory subunit (reviewed in reference 28). Thus, phase-specific fluctuations in the abundance of various cyclins constitute one mechanism by which the timing of cyclin-dependent kinase activation is controlled. It was shown that phase-specific transcriptional activation of cellular genes contributes to the control of cell cycle progression; in particular, heterodimeric transcription factor E2F/DP (referred to as E2F [17]) mediates the activation of several cellular genes at the G_1-to-S transition (27). More recently, it was shown that E2F binding sites determine activation at the G_1-to-S transition of the genes encoding E2F-1 (9, 11), a subunit of the E2F/DP heterodimer, and cyclin A (26), a key regulator of the G_1-to-S transition (24, 33), indicating a regulatory link between the ability of a cell to enter S phase and the appropriate activation of E2F during G_1.

Cyclin E (13, 18) is expressed in mid-G_1; it associates with and activates cdk2 kinase (6, 14). Conditional overexpression of cyclin E results in a decrease in the length of the G_1 interval, consistent with an acceleration of the G_1-to-S transition (25, 31). Microinjection of anti-cyclin E antibodies during G_1 inhibits entry into S phase in normal human fibroblasts (22), and genetic evidence suggests that cyclin E controls S phase entry during early embryogenesis in *Drosophila melanogaster* (12). These findings strongly suggest that cyclin E and its associated kinase are centrally involved in the decision to enter S phase in most if not all eukaryotic cells. Cyclin E mRNA is absent from serum-starved normal fibroblasts and appears in mid-G_1 (13). Regulation of cyclin E gene expression appears to be involved in the transformation of mammalian cells by viral oncogenes, since a rapid induction of cyclin E gene expression is observed after adenovirus E1A (29) or human papillomavirus type 16 E7 (32) is expressed in resting fibroblasts. In both cases, the ability of the viral oncogenes to activate cyclin E gene expression is genetically linked to their transforming potential. These results suggest that transcriptional regulation of the cyclin E gene contributes to the control of cell cycle progression in normal and virally transformed fibroblasts. Here, we report the cloning and initial characterization of the promoter and 5′ regulatory region of the murine cyclin E gene. It is shown that a 3.8-kb fragment contains all the *cis* elements required for G_1-specific expression of the gene and its activation by adenovirus E1A. An E2F site in the promoter which is required for activation of the gene in mid-G_1 was identified.

* Corresponding author. Mailing address: Deutsches Krebsforschungszentrum, FSP Angewandte Tumorvirologie, Abt. 0620, Im Neuenheimer Feld 242, D-69120 Heidelberg, Germany. Phone: 49-6221-424628. Fax: 49-6221-424902. Electronic mail address: P.Jansen-Duerr@dkfz-heidelberg.de.

MATERIALS AND METHODS

Cloning and DNA sequencing. A partial murine cDNA clone (1,661 bp) was isolated by low-stringency hybridization with a probe derived from human cyclin E cDNA (13). Genomic clones were isolated from a mouse liver genomic DNA library in the FIX vector (Stratagene) by using a 330-bp fragment derived from the 5′ end of the mouse cDNA clone as a probe. Sequencing of genomic DNA and cDNA was performed on double-stranded plasmid templates by the dideoxynucleotide chain termination method.

Cells and extracts. NIH 3T3 cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% calf serum (CS). Synchronous cell cycle progression was induced by serum readdition (10% CS) to cells which had been starved for 2 days in the presence of 0.5% CS (26). Synchrony was monitored by flow cytometry. Cellular extracts were prepared as described previously (26).

S1 mapping of transcriptional start sites. RNA was isolated from asynchronously growing NIH 3T3 cells and analyzed by S1 nuclease protection experiments as described previously (7). Plasmid pBCE(−3565/+513), containing the 5′-most 4,078 bp of the murine genomic DNA in Bluescript KS+ (Promega, Inc.), was linearized with *Bam*HI at nucleotide position 3660. The DNA was 5′ end labelled with T4 polynucleotide kinase and [γ-^{32}P]ATP. A double-stranded DNA fragment with a size of 638 bp that spanned positions 3022 to 3660 and that was labelled at position 3660 was obtained by cutting the linearized plasmid DNA with *Sty*I at position 3022. The 638-bp fragment was heat denatured for 5 min at 90°C; 40 μg of RNA was then added. The DNA-RNA mix was hybridized for 12 h at 58°C and incubated with 600 U of S1 nuclease (Promega) for 1 h at 25°C. After purification, the protected DNA fragments were analyzed on a 6% polyacrylamide gel that included 7 M urea.

Reverse transcriptase mapping of transcriptional start sites. The 5′ ends of cyclin E mRNA species were analyzed with the avian myeloblastosis virus reverse

transcriptase primer extension system (Promega). Primers derived from the exon sequences of the genomic fragment were 5′ end labelled with T4 polynucleotide kinase and hybridized to cellular RNA (20 μg per reaction) isolated from growing NIH 3T3 cells. The DNA fragments resulting from reverse transcription were analyzed on an 8% denaturing polyacrylamide gel containing 7 M urea and 1× Tris-borate-EDTA (TBE) buffer. The following primers were used: primer 3637/3663, 5′-ATCCGCGCCTGCCCCCTACACCGCGCT-3′, spanning positions 3637 to 3663 (exon 1); primer 4412/4436, 5′-TTCTTTCATGTTGCTGTGGTC CGT-3′, spanning positions 4412 to 4436 (exon 2); and primer 4277/4302, 5′-CTCTCCCTTGGCATGGCTCAGGACTT-3′, spanning positions 4277 to 4302 (exon 2).

Start site mapping by in vitro transcription analysis. In vitro transcription was performed with the HeLaScribe nuclear extract transcription kit (Promega Inc.) by following a procedure described by the supplier. Two micrograms of plasmid pBCE(−3565/+845) containing the 5′-most 4,410 bp of the murine genomic DNA in Bluescript KS+ (Promega, Inc.) was incubated with 10 to 15 μl of HeLa cell nuclear extract, and the transcription reaction was performed for 30 min at 30°C. RNA was purified and analyzed by primer extension as described above. The primer (5′ GGT CCG GTC TGG TCG GAC CCG GTC 3′) used for this experiment spans positions 3848 to 3825 in the genomic DNA.

Reporter plasmids and expression vectors. Fragments of the genomic clone obtained by digestion with various restriction enzymes were recloned by standard techniques into promoterless vector pXP1 (20) to produce reporter gene constructs pCE(−3565/+845), pCE(−3565/+263), pCE(−3565/+59), pCE(−3565/−50), pCE(−3565/−614), pCE(−543/+263), pCE(−94/+263), pCE(+95/+263), and pCE(+167/+263). The numbers in parentheses indicate the nucleotide positions with respect to the major transcriptional start site (see Fig. 2). By oligonucleotide reconstitution, the E2F consensus sequence was altered in construct pCE(−543/+263) to yield a *Hin*dIII recognition sequence without changing the adjacent promoter sequences. The resulting construct was designated pCE(−543/+263)ΔE2F.

Transfection experiments. Cells were transfected by the calcium phosphate method as described previously (26). Cells were harvested at 36 h posttransfection and assayed for reporter gene activity. Variations in transfection efficiency were corrected by normalizing luciferase activity to β-galactosidase activity expressed from a cotransfected cytomegalovirus-driven β-galactosidase reporter gene (26).

Bandshift experiments. A double-stranded oligonucleotide encompassing the E2F binding site of the cyclin E promoter (5′-GATCCGGGCGGGCGC GAGGGCGGGA-3′ and 5′-GATCTCCCGCCCTCGCGCCCGCCCG-3′) was 3′ end labelled and incubated with cellular extracts as described previously (23). The probes containing the E2F binding site of the adenovirus E2 promoter were described elsewhere (26). The E2F bandshift gels were run in a way to ensure maximum resolution, which precludes the retention of the free probe in the gel. The same amounts of free probe were present in all lanes, and the amounts were controlled by scintillation counting of the samples prior to loading (26). E2F-associated proteins were analyzed by incubation of the bandshift mixtures with specific antibodies on ice for 50 min prior to electrophoresis. p107 was detected with monoclonal antibody SD15 (a gift from N. Dyson, Charlestown, Mass.), and DP-1 was detected with a polyclonal antiserum (a gift from N. La Thangue, Glasgow, Scotland). Antibodies to pRb and p130 were purchased from Santa Cruz, Inc.

RESULTS

Cloning of the 5′ end of the mouse cyclin E gene. A mouse cyclin E cDNA clone was isolated from a BALB/c murine cDNA library (10) by using the human cyclin E cDNA as a probe under low-stringency-hybridization conditions. A cDNA fragment with a size of 1,661 bp was obtained and sequenced. The cDNA obtained was similar to a murine cyclin E cDNA clone described previously by others (4), but the cDNA obtained by us was missing the first 124 nucleotides. For the overlapping part, both sequences are identical, except that the sequence published by Damjanov et al. (4) contains an extra G at position 1238 which is not present in our cDNA clone. This insertion leads to a shift in the reading frame (Fig. 1A) and a considerable alteration of the C-terminal part of the predicted protein. This difference may explain why Damjanov et al. (4) found only relatively weak homology between the human and mouse cyclin E polypeptides (see Discussion). The 5′ part of the murine cyclin E cDNA was used to isolate genomic fragments from a mouse liver genomic library. The sequences of several overlapping fragments were determined by dideoxy sequencing of both strands. The complete nucleotide sequence was obtained for a contiguous DNA fragment with a size of 8,852 bp. A comparison with the cDNA sequence of murine cyclin E compiled from our own data and with the sequence published by Damjanov et al. (4) revealed the exon-intron structure of the murine cyclin E gene. These data define three

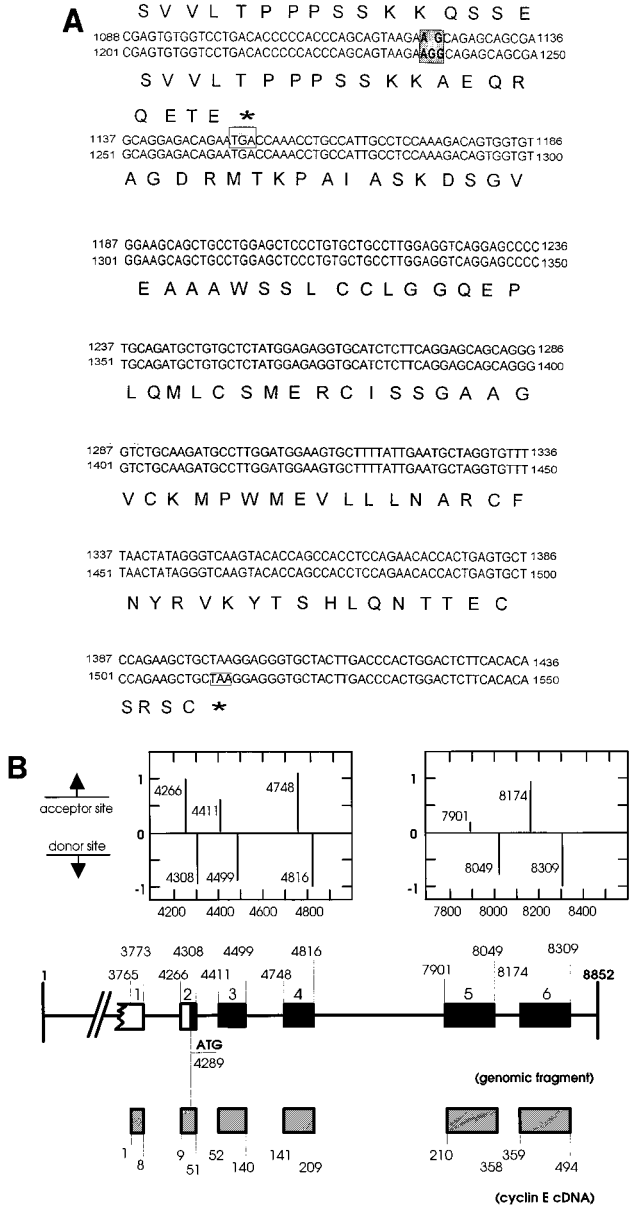


FIG. 1. Characterization of the murine cyclin E gene. (A) Alignment of the murine cyclin E cDNA sequence reported on here (upper sequence) with the sequence reported by Damjanov et al. (4) (lower sequence). Numbers refer to the cloned cDNAs described here and in reference 4. At position 1238, the latter sequence contains an additional G nucleotide, leading to a shift in the reading frame. In both cases, the predicted amino acid sequences are shown. As a consequence of the altered reading frame, the translational stop codon at position 1264 is neglected, resulting in the prediction of 82 additional amino acids. (B) Exon-intron structure. In the upper panel, the predicted splice donor and acceptor sites for the genomic fragment, as revealed by the DIANA program, are indicated. The structure of the genomic fragment is shown in the middle panel; coding exons are represented as solid boxes, and uncoding exons are shown as open boxes. The numbering refers to the nucleotide position in the genomic fragment, with the 5′-most nucleotide being 1. The hatched boxes (lower panel) indicate the sequence of the mouse cyclin E cDNA as compiled from own sequencing data and the sequence reported by others (4).

additional exons of the murine gene compared with the published cDNA sequence of human cyclin E (13). Of these additional exons, two contribute additional coding sequences, indicating that the open reading frame extends 5′ to the translation start site shown for human cyclin E. The 5′-most exon also contributes additional 5′ uncoding cDNA sequence. The predicted gene structure is shown in Fig. 1B. A prediction of the exon-intron structure of the genomic fragment was independently derived with the DIANA program (DNA Interactive Artificial Neural Network Analysis [7a]). In this program, which is a modified version of a program initially developed by Brunak et al. (2), artificial neural networks are used to predict potential splice sites with a very low error rate. As can be seen from Fig. 1B, the predicted splice sites were in perfect agreement with the locations of the exon-intron boundaries, as derived by a comparison of genomic and cDNA sequences.

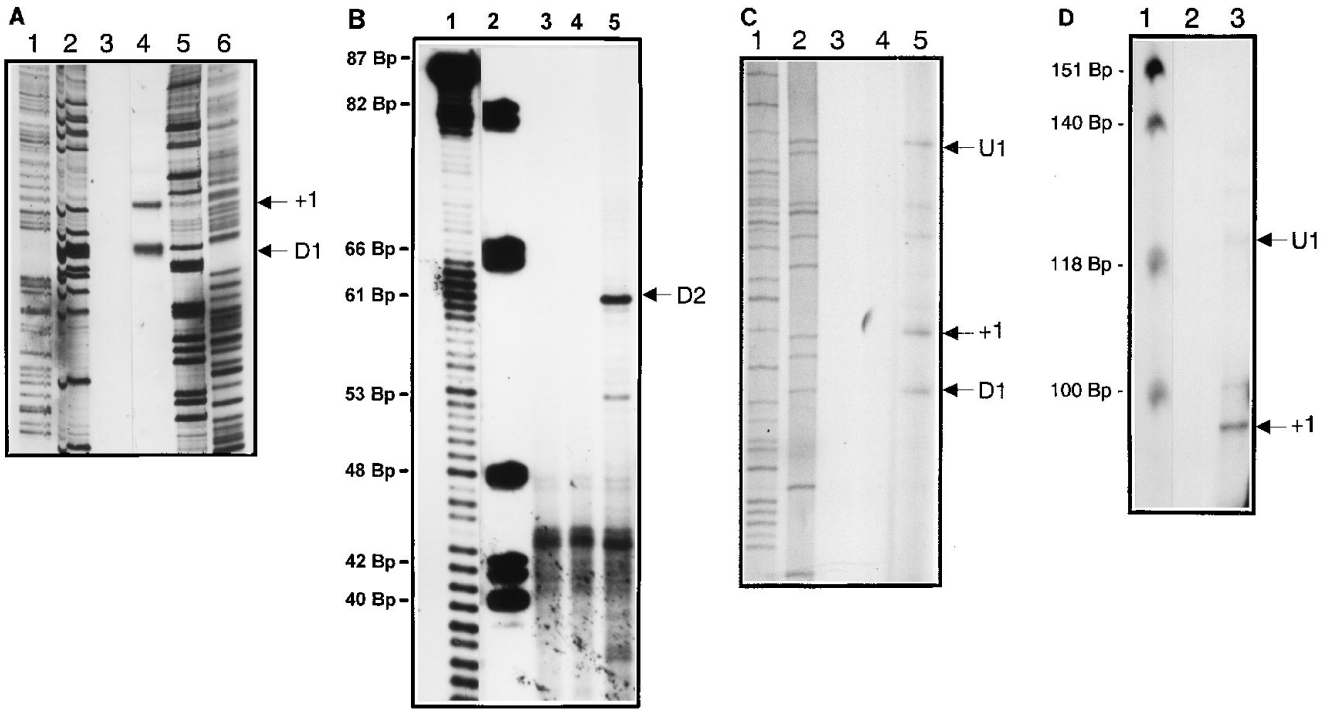
Identification of transcriptional start sites. The genomic fragment contains 3,765 bp 5′ of the first nucleotide of the cDNA isolated by Damjanov et al. (4). Since we cannot exclude the presence of additional introns 5′ of exon 1 (Fig. 1B), we first analyzed RNA produced by an in vitro transcription reaction. The failure of nuclear extracts to support mRNA maturation warrants the predominance of unspliced precursor RNA in such extracts, which then allows the unambiguous definition of the start site of a given transcription reaction (9). In vitro transcription was performed in a HeLa nuclear extract with plasmid pBCE(−3565/+845), which contains the 5′ part of the genomic sequence isolated by us (see Materials and Methods) (Fig. 1), being used as the template. The RNA produced by this reaction was analyzed by reverse transcription, with a primer (3825/3848) hybridizing to a sequence which is 84 bp downstream of the first nucleotide of the known cyclin E cDNA and which corresponds to sequences in the first known intron being used. The primer extension products were analyzed on a polyacrylamide gel, with a sequencing reaction product of the genomic clone reaction driven by the same primer being used as a size standard. This experiment revealed two major bands with sizes of 272 and 283 bp (Fig. 2A), indicating that the transcripts were initiated at positions 3565 (designated +1) and 3576 (designated D1 [downstream start site 1]), respectively.

To characterize the 5′ ends of cyclin E mRNA species from murine cells, RNA was prepared from proliferating NIH 3T3 cells and analyzed by primer extension. Since only 9 bp of exon 1 are known from the cDNA analysis (Fig. 1B), a primer which extends from nucleotides 4277 to 4302 and which is complementary to sequences in exon 2 was used in the first experiments. Reverse transcription reproducibly yielded a major extension product with a size of 61 bp (Fig. 2B) and several minor products. This result localizes a transcriptional start site (designated D2 [downstream start site 2]) to position 3756 of the genomic fragment; this conclusion was confirmed by a similar experiment using a second primer extending from nucleotides 4412 to 4436 (data not shown). Our inability to visualize start sites +1 and D1, as revealed by the analysis of in vitro-transcribed RNA, in these experiments might be due to the limited processivity of the reverse transcriptase, given the fact that the genomic DNA fragment analyzed in this report has a high GC content, which is known to limit the processivity of most reverse transcriptases. In an attempt to confirm start sites +1 and D1, the primer extension analysis of cellular RNA was repeated with a new primer (3637/3663) that is located farther upstream. In support of the assumption that additional transcriptional start sites are located 5′ of position 3756, several extension products were obtained with this new primer; major fragments with sizes of 87 and 98 bp were identified (Fig. 2C). The start sites identified by two of these fragments colocalize with sites +1 and D1 identified with in vitro-transcribed RNA. A third major fragment with a size of 127 bp was also observed in these experiments, revealing an additional start site (referred to as U1 [upstream start site 1]) at position 3536. To further characterize the 5′ end of the cyclin E mRNA in murine cells, RNA from proliferating NIH 3T3 cells was analyzed by an S1 nuclease protection assay. To this end, a 638-bp fragment was prepared, labelled at the *Bam*HI site at position 3660, and hybridized to cellular RNA. While this strategy precludes detection of the start site at position 3756, the fragment was designed to reveal the start sites identified in the previous experiments. A major protected fragment with a size of 95 bp was obtained by S1 nuclease analysis, revealing a major transcriptional start site at position 3565 which coincides with start site +1 as identified by the other two methods.

Taken together, the results obtained allow the following conclusions. The start site at position 3565 is revealed as a clear band by all three methods and was designated +1. An additional start site was mapped to position 3576 by in vitro transcription (Fig. 2A) and primer extension of cellular RNA (Fig. 2C); this site, designated D1 (downstream site 1), was also revealed as a weak band by the S1 nuclease protection assays (data not shown). An additional start site at position 3536, designated U1, was revealed by analysis of cellular RNA by both S1 nuclease protection (Fig. 2D) and primer extension analyses (Fig. 2C), but this site was not efficiently used in the in vitro transcription reaction (Fig. 2A). Several additional bands were apparent in the experiments shown in Fig. 2; their relatively low abundances indicate that if they represent transcriptional initiation sites, these sites only marginally contribute to promoter activity. The start site at position 3756, designated D2 (for downstream initiation site 2) in Fig. 2B, could be clearly demonstrated by primer extension analysis only; however, transfection studies performed with various reporter gene constructs suggest that this site contributes to promoter activity in NIH 3T3 cells (see below).

Functional analysis of the promoter elements contained in the 3.8-kb genomic fragment. To analyze the ability of the genomic fragment identified in this report to serve as promoter for RNA polymerase II, a reporter gene construct designated pCE(−3565/+263) was prepared by inserting the 3.8-kb genomic fragment in front of the firefly luciferase cDNA in vector pXP1 (20). Upon transfection into asynchronously growing NIH 3T3 cells, this construct showed relatively strong luciferase activity, comparable to the activity obtained with several cellular and viral promoters, including the human cyclin A promoter (data not shown) (see also Fig. 4B). For further analysis of the cyclin E promoter fragment, additional deletion mutants were prepared (Fig. 3). From these experiments, it appears that sequences between −543 and +263 retain full promoter activity in asynchronous cells. Further deletion to position −94 still leaves more than 50% of promoter activity intact; thus, construct −94/+263 can be considered a minimal promoter containing all detectable transcriptional start sites. As was expected, deletion of major start sites U1, +1, and D1, as in construct +95/+263, led to a severe reduction of promoter activity. However, pCE(+95/+263) still retains roughly 10% of basal promoter activity (Fig. 3) and still responds to E1A (see below), indicating that transcriptional start site D2 is used in NIH 3T3 cells.

Regulation of promoter activity during the cell cycle and by E1A. To analyze if the promoter fragment described in this communication contains the *cis*-acting elements required for cell cycle regulation of the cyclin E gene, NIH 3T3 cells were transfected with construct pCE(−3565/+263); transfected cells



E

-144 CACGCCCGTAAAAGAACACGCCCCCCGGGAGGCCACGCCCCCACCAGAGCTCCTCGCTGG -85
GTGCGGGCATTTTCTTGTGCGGGGGGCCCTCCGGTGCGGGGGTGGTCTCGAGGAGCGACC

U1

-84 TCCGCGCGGCGACGGGGCGGGCCCGGGCCAGCGGCGGCCGCTGAGGGGCTCGCAGCCCTC -25
AGGCGCGCCGCTGCCCCGCCCGGGCCCGGTCGCCGCCGGCGACTCCCCGAGCGTCGGGAG

E2F I +1 D1

-24 GGGCGGGCGCGAGGGCGGGACGGGGCCGGTGCCGCGCGCTGGGATTTTTAAATGTCCCGC +36
CCCGCCCGCGCTCCCGCCCTGCCCCGGCCACGGCGCGCGACCCTAAAAATTTACAGGGCG

+37 TCGAAGCCGGGCGCAGGAGCAGCCGGCTCGACAGCCAGCGCGGTGTAGGGGGCAGGCGCG +96
AGCTTCGGCCCGCGTCCTCGTCGGCCGAGCTGTCGGTCGCGCCACATCCCCCGTCCGCGC

+97 GATCCCGCCAGCGCCGCGGCGGCCGCCGCTTCCCGCCTCCTGCTTCCCGCTCGCCGTCGG +156
CTAGGGCGGTCGCGGCGCCGCCGGCGGCGAAGGGCGGAGGACGAAGGGCGAGCGGCAGCC

D2

+155 CCTCCTGCCCGCTCGAGCGCGCCTGCCTGCGGCGGACACAGCTTCGGGTACGGGCTGCCT +216
GGAGGACGGGCGAGCTCGCGCGGACGGACGCCGCCTGTGTCGAAGCCCATGCCCGACGGA

+217 GGACTCCCGGGACTCCCGGGACTCCCACGACTCCCAGGCGTCCGGACCGGGGTCCGACCAG +276
CCTGAGGGCCCTGAGGGCCCTGAGGGTGCTGAGGGTCCGCAGGCCTGGCCCAGGCTGGCC

FIG. 2. Mapping of the transcriptional start sites of the murine cyclin E promoter. (A) Primer extension with in vitro-transcribed RNA. In vitro transcription was performed with construct pBCE(−3565/+845) being used as a template in a nuclear HeLa extract. Transcripts were hybridized to a primer extending from nucleotides 3825 to 3848 (Fig. 1B). After the reverse transcriptase reaction, the resulting fragments were run on a 7 M urea–6% polyacrylamide gel together with a sequencing reaction product from a reaction performed with the same primer. Start sites were deduced on the basis of the adjacent nucleotides of the sequencing reaction product in the gel. The sequencing ladder is given in lanes 1, 2, 5, and 6. Lane 4 shows the primer extension with the in vitro-transcribed cyclin E promoter transcripts; lane 3 represents a negative control with yeast tRNA. Major extension products were named +1 and D1. (B) Primer extension of cellular RNA with primer 4277/4302. RNA from proliferating NIH 3T3 cells was analyzed by primer extension with avian myeloblastosis virus reverse transcriptase. An oligonucleotide corresponding to the genomic sequence between 4277 and 4302 (Fig. 1B) was used as a primer. Extension products are shown in lane 5. Control reactions in which cellular mRNA was replaced with yeast tRNA are shown in lanes 3 and 4. Lane 1 represents a reverse transcriptase analysis of kanamycin mRNA (Promega), which was used as a positive control; lane 2 contains 5′-end-labelled restriction fragments of ϕX174 DNA cleaved with *Hin*fI. Fragment sizes are given in base pairs. (C) Primer extension of cellular RNA with primer 3637/3663. RNA from proliferating NIH 3T3 cells was analyzed by primer extension as described for panel B, except that a primer extending from nucleotides 3677 to 3663 was used. Primer extension results in three major products, as indicated by arrows (lane 5). Lanes 1 and 2 show a sequencing ladder from a sequencing reaction performed with the same primer. Yeast tRNA was used as a negative control (lanes 3 and 4). (D) S1 mapping of cellular RNA. RNA was prepared from asynchronously growing NIH 3T3 cells and hybridized to a ^{32}P-labelled DNA fragment of the murine cyclin E promoter stretching from −543 to +95 as described in Materials and Methods. S1 nuclease digestion of the DNA-RNA hybrid yielded fragments with lengths of 95 and 124 bp. The fragments are indicated by arrows and are named D1 and +1, respectively. (E) Location of the major and minor transcriptional start sites of the murine cyclin E promoter. The locations of major and minor transcriptional start sites, as deduced from experiments reported on in panels A through D, are marked by arrows. The E2F consensus sequence is shown as shaded box (see text). The numbering of the nucleotides relates to the major transcriptional start site at +1.

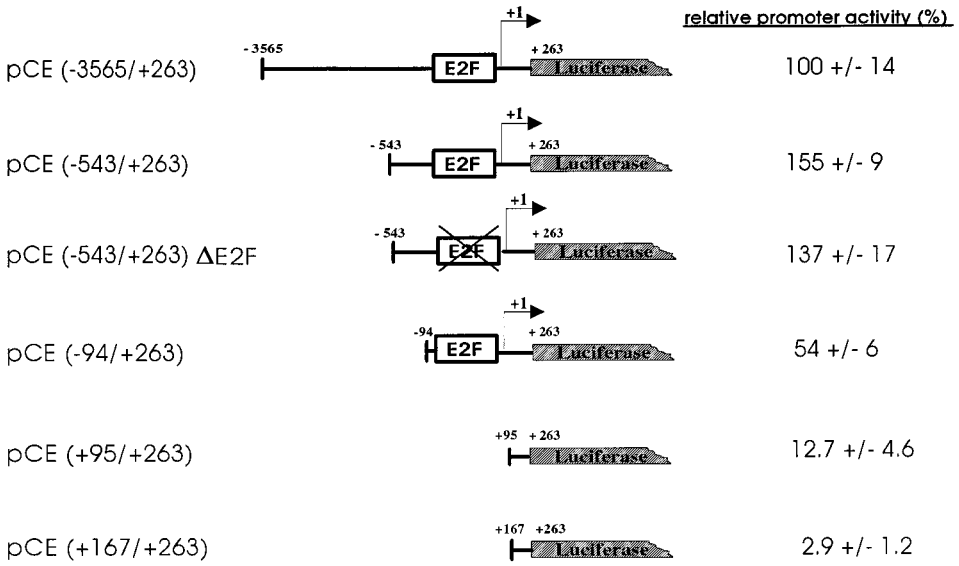


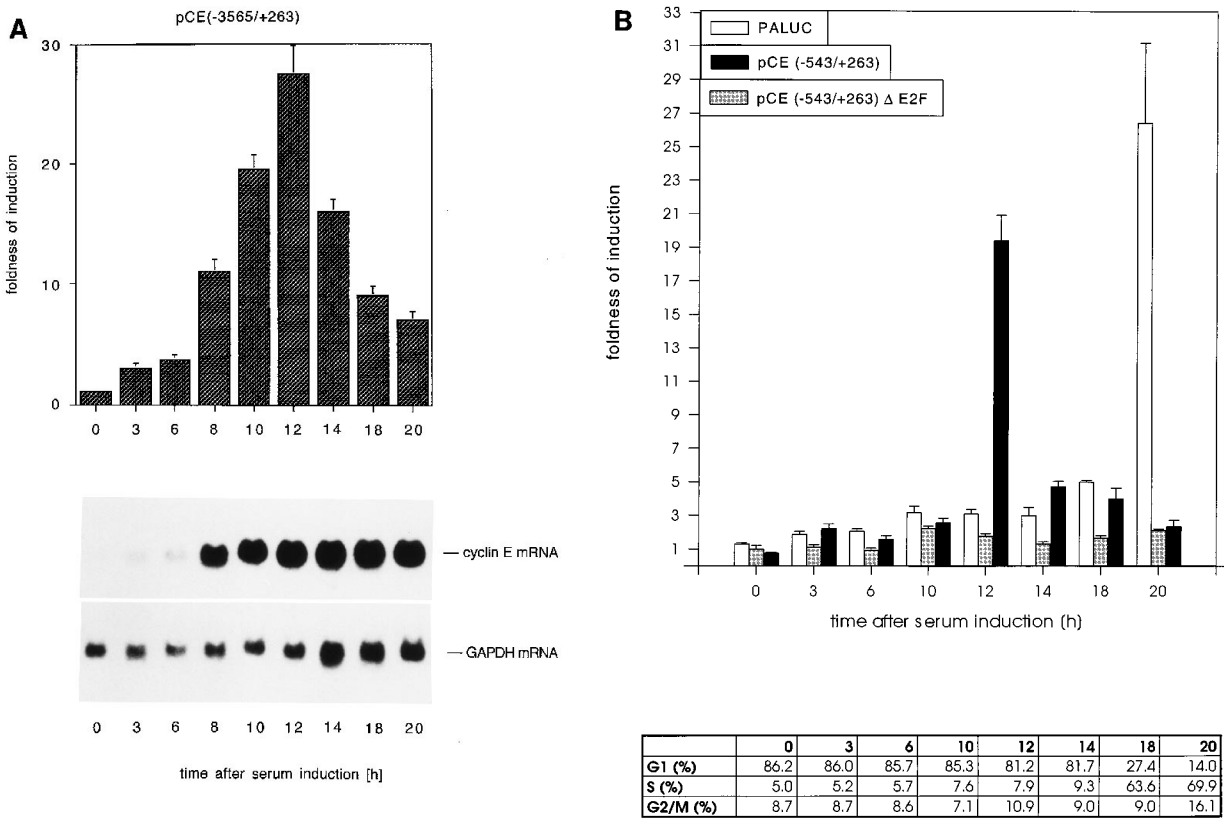
FIG. 3. Activities of cyclin E promoter-derived reporter constructs. The structures of the different 5′ deletion constructs and the point mutations of the murine cyclin E promoter are shown along with the basal promoter activities measured in transient transfection experiments with asynchronously growing NIH 3T3 cells. Transfections were performed and analyzed as described in the legend to Fig. 4. The mean values of at least four experiments are given.

were synchronized by keeping them in the presence of 0.5% CS for 72 h and by a subsequent readdition of 10% CS. Progression through the cell cycle was monitored by flow cytometry. At regular intervals after serum readdition, samples were taken and reporter gene activity was analyzed by measuring luciferase activity. The promoter activity of pCE(−3565/+263) was relatively low in G_0 cells and was increased about 30-fold at 12 h after serum addition; a considerable decrease in luciferase activity is noted at later time points. Under the experimental conditions used here, cells enter S phase with high synchrony between 14 and 18 h (data not shown). These data indicate that the 3.8-kb genomic fragment confers transcriptional activation during mid-G_1 in synchronized NIH 3T3 cells, similar to the expression profile of the endogenous cyclin E gene in these cells, as shown by Northern (RNA) blot analysis (Fig. 4A).

It was shown that expression of cyclin E is strongly induced by adenovirus E1A in both human and rodent cells (29). To analyze whether the cyclin E promoter fragment isolated in this study contains the *cis* regulatory elements required for induction by E1A, NIH 3T3 cells were transfected with cyclin E promoter-reporter genes and an expression vector for the 12S form of adenovirus E1A which had been shown to be sufficient to induce cyclin E gene expression in adenovirus-infected rodent cells (28a). Cotransfection of pCE(−543/+263) with E1A resulted in a 15-fold ± 2-fold increase of reporter gene activity, indicating that this fragment retains essential *cis* elements required for promoter activation by E1A. For the initial characterization of the E1A-responsive elements in the cyclin E promoter, the deletion mutants described in Fig. 3 were used. A gradual decrease of responsiveness to E1A was observed for promoter sequences with progressive deletions from the 5′ end. While reporter gene constructs pCE(−94/+263) and pCE(+95/+263) are still significantly inducible by E1A (13-fold ± 1.5-fold and 8.2-fold ± 1-fold inductions, respectively), the E1A responsiveness is lost by further 5′ deletion to position +167 (1.9-fold ± 0.3-fold induction). These results suggest the presence of several distinct E1A-responsive elements in the murine cyclin E gene.

Cell cycle regulation of the cyclin E promoter depends on an E2F binding site. Phase-specific promoter activity was retained in a fragment extending from −543 to +263 [designated pCE(−543/+263)] (Fig. 4B), indicating that this fragment contains the essential elements required for cell cycle regulation of the murine cyclin E gene. There is a sequence with homology to a binding site for transcription factor E2F (17) located at positions −13 to −18 in the murine cyclin E promoter (Fig. 2E). E2F was shown to contribute to the transcriptional regulation of several genes activated in G_1 (for a recent review, see reference 15), including the human cyclin A gene (26). To analyze the role of the putative E2F site in the control of cyclin E gene expression, clustered point mutations (changing CTCGCG to AAGCTT) were generated in the E2F homology region in construct pCE(−543/+263) to yield construct pCE(−543/+263)ΔE2F. Deletion of the E2F consensus sequence led to a slight reduction of promoter activity, as measured in asynchronously growing NIH 3T3 cells (Fig. 3). After these constructs were assayed in synchronized NIH 3T3 cells, a remarkable difference was observed. While both constructs displayed similar activities throughout most of the cell cycle, the 20-fold induction of promoter activity observed with the wild-type construct at 12 h after serum stimulation was not detected with the mutated reporter gene (Fig. 4B). These findings imply that the E2F consensus sequence is required for the timing of cyclin E gene activation in mid-G_1. For comparison, cells were also transfected with a reporter gene construct (PALUC [8]) containing the cell cycle regulatory elements of the human cyclin A promoter. Activation of the cyclin E promoter precedes activation of the cyclin A promoter by about 6 h in these experiments (Fig. 4B), roughly paralleling the accumulation patterns of the respective mRNAs in synchronized NIH 3T3 cells (Fig. 4A and data not shown).

To address the question of which cellular proteins bind to the promoter element defined by the experiments reported on in Fig. 4B, bandshift analysis was carried out with a double-stranded oligonucleotide (referred to as cycEI carrying the putative E2F binding site of the cyclin E promoter. In extracts of asynchronously growing NIH 3T3 cells, several complexes which competed with the homologous probe and an oligonucleotide (E2wt) containing the wild-type E2F binding site from the adenovirus E2 promoter but not with a mutated version of



	0	3	6	10	12	14	18	20
G1 (%)	86.2	86.0	85.7	85.3	81.2	81.7	27.4	14.0
S (%)	5.0	5.2	5.7	7.6	7.9	9.3	63.6	69.9
G2/M (%)	8.7	8.7	8.6	7.1	10.9	9.0	9.0	16.1

FIG. 4. Cyclin E promoter activity in synchronized NIH 3T3 cells. (A) Serum induction of cyclin E promoter activity. NIH 3T3 cells were transfected with promoter-reporter construct pCE(−3565/+263) and were subsequently serum starved (0.5% CS) for 72 h. Cell proliferation was induced by the addition of Dulbecco's modified Eagle's medium containing 10% CS, and cells were harvested at the indicated time points (in hours) for assays of luciferase and β-galactosidase activity. Luciferase activity was normalized to the β-galactosidase activity of a cotransfected cytomegalovirus-driven β-galactosidase vector serving as an internal control and is given as fold induction. The mean values of four experiments are shown. At each time point, RNA was extracted from parallel samples and analyzed by Northern blotting for expression of the endogenous cyclin E gene. Reprobing for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA was performed as a loading control. (B) Cell cycle regulation of the murine cyclin E promoter depends on an E2F binding site. NIH 3T3 cells were transfected with promoter and reporter constructs pCE(−543/+263) and pCE(−543/+263)ΔE2F and were subsequently serum starved (0.5% CS) for 72 h. Cell proliferation was induced and reporter gene activities were determined as described for panel A. In parallel experiments, cells were transfected with construct PALUC containing the human cyclin A promoter (8). Fluorescence-activated cell sorter analysis was used to assess progression through the cell cycle. The fold induction is given. The mean values of four independent experiments are shown. Error bars show standard deviations.

this oligonucleotide (E2mut) in which the E2F consensus sequence had been destroyed (Fig. 5A) were obtained. All complexes obtained with the cycEI oligonucleotide were supershifted by the addition of an antibody to DP-1, indicating that DP-1 is a major component of the complexes shown in Fig. 5A. Complexes I and II were supershifted by antibodies to pRb-related protein p107, indicating that these complexes contain p107. Antibodies to pRb and p130, used as controls here, did not react strongly with any complex shown in Fig. 5A, consistent with the observation that complexes of E2F with pRb and/or p130 are hardly detectable in extracts of growing NIH 3T3 cells (16, 26). The complexes labelled III, IV, and V in Fig. 5A were not affected by antibodies to either pRb family member, indicating that these complexes may represent different forms of free E2F. While we were unable to identify the E2F family members present in these complexes because of the lack of specific supershifting antibodies, the data clearly establish the promoter sequence contained in oligonucleotide cycEI as a bona fide E2F binding site.

To analyze the functional consequences of the binding of E2F/DP complexes to this element, NIH 3T3 cells were transfected with cyclin E promoter and reporter gene constructs and expression vectors for DP-1 and E2F-4, a p107-binding member of the E2F family of proteins (17). As can be seen from Fig. 5B, ectopic expression of E2F-4/DP-1 led to the transcriptional activation of reporter gene construct pCE(−543/+263). Construct pCE(−543/+263)ΔE2F, in which the E2F site has been destroyed, responded much less to the ectopic expression of E2F-4/DP-1. Thus, E2F can bind to the cyclin E promoter via this element and subsequently activate transcription.

DISCUSSION

Exon-intron structure of the murine cyclin E gene. Results presented in this communication demonstrate that we have isolated a genomic fragment containing the complete coding sequence of the murine cyclin E gene upstream of exon 6. In agreement with the characterization of a murine cyclin E cDNA published by others (4), the mouse cyclin E cDNA has additional 5′ sequence which is missing in the human cyclin E cDNA reported on by Koff et al. (13). While Damjanov et al. (4) found only 67% homology between cyclin E sequences from humans and mice at the amino acid level, the cDNA sequence reported here yields a sequence identity of 81% and

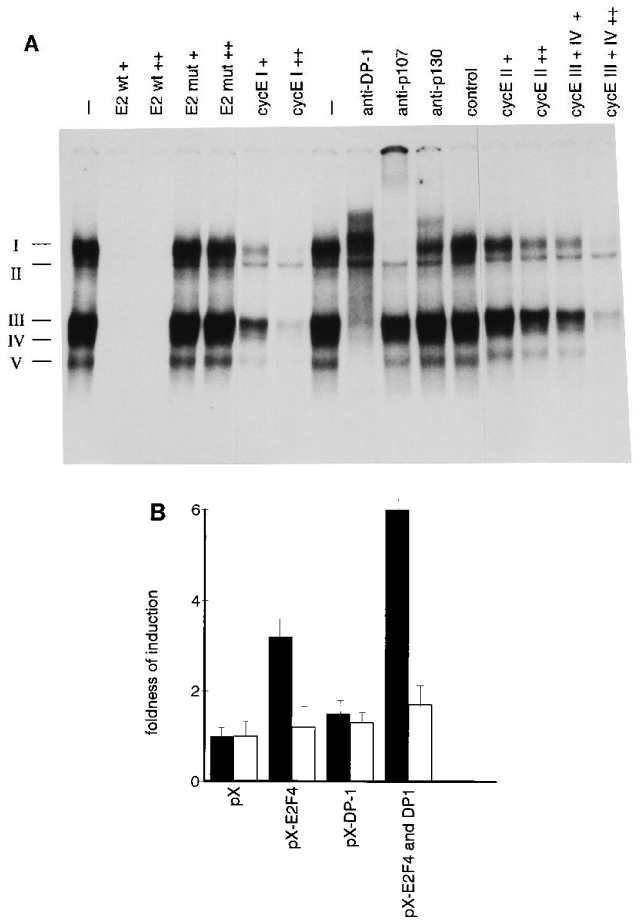


FIG. 5. E2F binding to and E2F activation of the cyclin E promoter. (A) E2F binding to the murine cyclin E promoter. A 3′-end-labelled, double-stranded oligonucleotide [cycE(−25/−5)] carrying the E2F consensus sequence (E2FI) of the cyclin E promoter was incubated with an extract of asynchronously growing NIH 3T3 cells and analyzed by native gel electrophoresis. Specificity of binding was assessed by competition with excess unlabelled homologous probe (cycEI) and oligonucleotides containing the wild-type (E2 wt) or mutated version (E2mut) of the E2F binding site from the adenovirus E2 promoter. Oligonucleotides corresponding to the E2FII (+22 to +46) and E2FIII+IV (+121 to +150) sequences of the murine cyclin E promoter were used for comparison. Specific complexes are indicated at the left. Antibodies to p107, p130, DP-1, and pRb (control) were added as indicated. (B) Activation of the cyclin E promoter by E2F-4/DP-1. Constructs pCE(−543/+263) (solid bars) and pCE(−543/+263)ΔE2F (open bars) were transiently transfected in growing NIH 3T3 cells along with expression vectors for E2F-4 and DP-1 as indicated. Promoter activity was determined as described in the legend to Fig. 4. The fold induction is shown. Error bars show standard deviations.

a similarity of 90% for human and mouse cyclin E sequences at the amino acid level (data not shown). This discrepancy is explained by a shift in the reading frame due to the insertion of an extra G at position 1238 in the cDNA sequence published by Damjanov et al. (4) (Fig. 1A). While these authors deduced from their sequencing data that the open reading frame has a size of 491 amino acids, the data shown here indicate that the cDNA of murine cyclin E encodes a protein with a size of 409 amino acids. The additional C-terminal 82 amino acids are also missing from the human cyclin E polypeptide. While it is possible that the cDNA cloned by Damjanov et al. (4) corresponds to a naturally occurring variant of cyclin E, the high degree of homology between the proteins coded for by the human cDNA cloned by Koff et al. (13) and the cDNA sequence reported here indicates that this may be the regular form of murine cyclin E.

Mapping of transcriptional start sites. The genomic fragment we have isolated contains a 3.8-kb segment which is upstream of the 5′ end of the published cDNA sequence and which potentially contains the promoter and additional upstream regulatory elements. Using three different experimental approaches, we have determined the predominant 5′ ends of both cyclin E mRNA from NIH 3T3 cells and unspliced cyclin E RNA produced by in vitro transcription in order to define the principal transcriptional start sites in the mouse cyclin E gene. Comparing the results obtained in these different experiments requires a reliable method for determining the exact size of particular DNA fragments. High-resolution mapping of primer extension products was performed by using sequencing reaction products from reactions performed with the same primer as size standards. Similarly, the products of S1 protection analysis were analyzed by comparison with the products of a sequencing reaction of the genomic DNA. While the gel shown in Fig. 2D contains a commercially available molecular weight standard, the actual size determination of the protected fragments was performed by comparing them with a sequencing ladder generated by primer 3637/3663 (data not shown). Several distinct transcriptional start sites of different abundances were delineated by this analysis. The start site at position 3565 is revealed as a clear band by all three methods and was designated +1. An additional start site was mapped to position 3576 by in vitro transcription and primer extension of cellular RNA (Fig. 2A and C); this site, designated D1, was also revealed as a weak band by the S1 nuclease protection assays (data not shown), indicating that it represents a bona fide start site. An additional start site at position 3536, designated U1, was revealed by both S1 nuclease protection analysis and primer extension analysis of cellular RNA.

In addition to the three potential start sites described above, we obtained evidence for an additional start site located farther downstream. After RNA from proliferating NIH 3T3 cells was analyzed by primer extension with a primer which is complementary to sequences in exon 2, one major extension product with a size of 61 bp was obtained, indicating a potential transcriptional start site at position 3756 of the genomic fragment. Our inability to detect major start sites U1, +1, and D1 in this experimental setting could be due to the limited processivity of the reverse transcriptase, given the fact that this part of the mouse genomic DNA has a high GC content, which is known to limit the activity of the enzyme. While this raises the possibility that the occurrence of the 61-bp fragment might reflect a premature termination of the reverse transcriptase rather than the existence of an mRNA species originating at position 3756, the strong and distinct band obtained in the experiment reported on in Fig. 2B further suggests that this extension product indeed represents the 5′ end of a cellular mRNA species. That an additional start site downstream of +95 is used in NIH 3T3 cells is further supported by transfection experiments reported on in Fig. 3, indicating that the presence of cyclin E genomic sequences between positions +95 and +263 is sufficient for the efficient transcriptional activation in *cis* of a promoterless luciferase reporter gene.

***cis*-acting elements required for regulation of cyclin E promoter activity.** The 3.8-kb fragment isolated as a result of this work confers activation in late G_1 on the luciferase reporter gene in synchronized NIH 3T3 cells, coinciding with the induction of endogenous cyclin E mRNA in these cells (Fig. 4A); this fragment also mediates activation of the reporter gene in response to adenovirus E1A, a known inducer of cyclin E gene expression (29). These findings support the conclusion that the

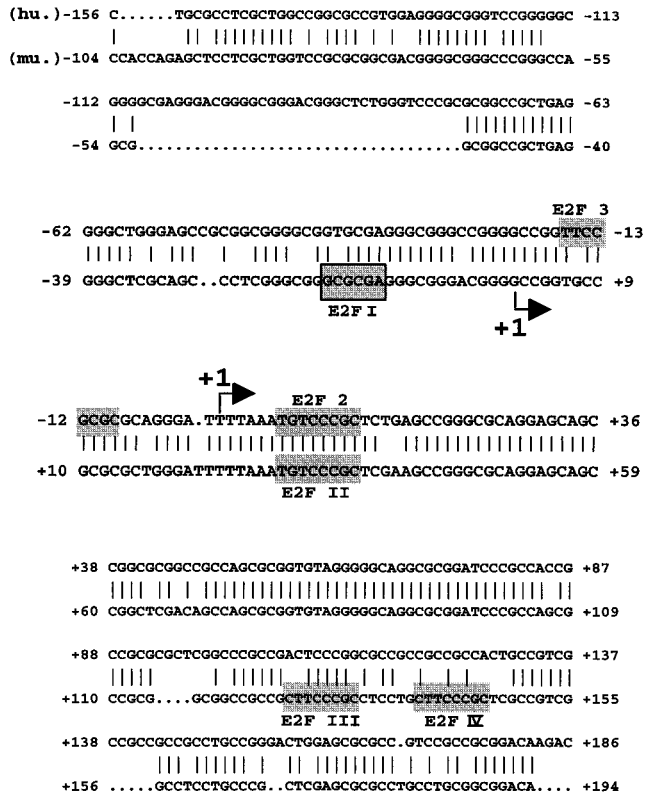


FIG. 6. Comparison of the cyclin E promoter regions of human and murine genes. The DNA sequences corresponding to the human (hu.) and murine (mu.) cyclin E promoters were aligned according to sequence homology. In the human DNA, potential E2F binding sites, as identified in reference 21, are highlighted. The E2F site shown in this report to contribute to cell cycle regulation of the murine gene is designated E2FI. Additional potential E2F binding sites in the murine promoter are marked E2FII, E2FIII, and E2FIV (see text). The major transcriptional start sites in either gene are marked by arrows.

essential regulatory elements of the murine cyclin E gene are present on this fragment. Cell cycle regulation of the promoter is retained in a fragment that has a size of 806 bp and that is present in construct pCE(−543/+263) (Fig. 4B). A potential binding site for the E2F transcription factor was identified in this promoter fragment by sequence comparison. Specific mutation of this site, as in construct pCE(−543/+263)ΔE2F, yielded a reporter gene that is no longer cell cycle regulated. Thus, this element is involved in G_1-specific activation of the cyclin E gene. We have shown that an oligonucleotide containing this element (cycEI) (Fig. 6) binds free E2F and some E2F-p107 complexes in extracts from NIH 3T3 cells (Fig. 5A). When oligonucleotide cycEI was added to bandshift reactions performed with a labelled oligonucleotide containing the E2F site from the adenovirus E2 promoter, we found that cycEI competes for binding, although with lower affinity than the E2-derived probe (data not shown). While these results indicate that the site designated E2FI binds E2F complexes with lower affinity than the site derived from the adenovirus E2 gene, the in vitro binding affinity of a given element for E2F family members is not strictly linked to the element's functional significance. For example, a low-affinity E2F binding site is essential for cell cycle regulation of the human cyclin A gene promoter (26); apparently, this variant E2F site cooperates with additional promoter elements to control cyclin A gene expression (34). On the other hand, it was reported that a high-affinity E2F binding site in the cdc2 promoter is irrelevant for cell cycle regulation of the cdc2 gene (30), indicating that the in vitro binding affinity of a given E2F site is no reliable parameter for the prediction of its function. Our demonstration that E2F/DP heterodimers are the major proteins that bind the cycEI element in cellular extracts and that elimination of this element abolishes cell cycle regulation of the promoter establishes this element as an additional low-affinity E2F binding site involved in the G_1 regulation of a cellular gene.

Whereas deletion of the E2F site rendered the promoter insensitive to cell cycle progression, the induction of cyclin E promoter activity by E1A was only moderately reduced upon mutation of the cycEI element (data not shown). Furthermore, construct pCE(+95/+263), from which cycEI has been deleted, retains responsiveness to E1A. These data indicate that several additional promoter elements may contribute to activation by E1A. The existence of multiple E1A-responsive elements in a single promoter has ample precedent in the literature (for a review, see reference 19). To address the possibility of additional E1A-responsive elements in the murine cyclin E promoter, the sequence was scanned for motifs resembling binding sites for known E1A-responsive transcription factors. Three additional E2F-like sequences were found and designated E2FII, E2FIII, and E2FIV (Fig. 6). After oligonucleotides containing these motifs were used as competitors in bandshift experiments using cycEI as the labelled probe, an oligonucleotide containing sites III and IV (cycEIII+IV) displayed binding activity similar to that obtained with oligonucleotide cycEI, while an oligonucleotide containing the E2FII motif (cycEII) displayed very weak binding activity (Fig. 5A). These results indicate that there are additional potential E2F binding sites in the murine cyclin E promoter. The failure of construct pCE(−543/+263)ΔE2F, which retained motifs cycEII, cycEIII, and cycEIV, to significantly respond to ectopic expression of E2F-4/DP-1 indicates that these elements are functionally different from site E2FI, as the latter contributes to promoter activation by ectopic expression of E2F-4/DP-1 (Fig. 5B). However, the present data do not exclude the possibility that these elements are involved in the E1A responsiveness of the cyclin E promoter.

Comparison of the cyclin E promoters from mice and humans. After this work was submitted, the cloning and initial characterization of the human cyclin E gene were reported on by Ohtani et al. (21). These authors reported that the human cyclin E promoter is induced by ectopic expression of E2F/DP proteins, and they found three potential E2F binding sites in the human cyclin E gene. On the basis of these data and our own findings, we have carried out an extensive comparative analysis of the genomic fragments from mice and humans. Although it is obvious from the sequences that homologous parts of the mouse and human genomes were cloned, there is a remarkable difference in the primary nucleotide sequences over long stretches (Fig. 6). Furthermore, the available evidence suggests considerable differences in the architectures of both promoters. First, the E2F binding site identified by us as being essential for cell cycle regulation of the murine gene is not conserved in the human promoter. Conversely, only one of the two potential E2F sites that jointly mediate a moderate negative regulation of the human promoter (referred to as site 2 in reference 21) is conserved in the mouse genome, and our analysis of the murine promoter did not reveal evidence for negative regulation. Second, the major transcriptional start sites are not conserved; by using three independent mapping strategies, we show that at least four distinct start sites distributed over more than 200 bp are used in the mouse cyclin E gene and that none of them coincides with the major start sites

described for the human promoter in the report of Ohtani et al. (21).

We can exclude any technical reasons for the observed sequence deviation, since the murine genomic DNA was independently isolated and sequenced several times; furthermore, we found a perfect alignment with the cyclin E cDNA in the coding region of the genomic fragment. There is a precedent for species-specific differences in the promoter organization of cellular G_1-to-S-specific genes. The promoter of the thymidine kinase gene is regulated through E2F-like sequences in mice and humans; nevertheless, a sequence comparison indicates considerable differences in the promoter architectures of different species (1). This view is strongly supported by the available functional data (3, 5). While more work is required to understand cell cycle regulation of the murine cyclin E promoter, it will be interesting to compare its regulation with that of the human cyclin E promoter once more information is available.

ACKNOWLEDGMENTS

We thank Michele Pagano, David Beach, Rene Bernards, and Nick La Thangue for the generous gifts of various antibodies and cDNA plasmids. We acknowledge Joseph Nevins for sharing information with us before publication.

Work in P.J.-D.'s laboratory was supported in part by a research grant from the Human Frontiers Science Program Organisation.

REFERENCES

1. **Arcot, S. S., D. V. Traina, and P. L. Deininger.** 1991. The rat thymidine kinase gene 5′ region: evolution of a promoter. DNA Seq. **2:**129–131.
2. **Brunak, S., J. Engelbrecht, and S. Knudsen.** 1991. Prediction of human mRNA donor and acceptor sites from the DNA sequence. J. Mol. Biol. **220:**49–65.
3. **Chang, Z. F., D. Y. Huang, and T. C. Lai.** 1995. Different regulation of the human thymidine kinase promoter in normal human diploid IMR-90 fibroblasts and HeLa cells. J. Biol. Chem. **270:**27374–27379.
4. **Damjanov, I., J. Shan, R. F. Wang, A. Damjanov, and J. A. Deloia.** 1994. Molecular cloning and characterization of murine cyclin E. Biochem. Biophys. Res. Commun. **201:**994–1000.
5. **Dou, Q. P., S. Zhao, A. H. Levin, J. Wang, K. Helin, and A. B. Pardee.** 1994. G1/S-regulated E2F-containing protein complexes bind to the mouse thymidine kinase gene promoter. J. Biol. Chem. **269:**1306–1313.
6. **Dulic, V., E. Lees, and S. Reed.** 1992. Association of human cyclin E with a periodic G1-S phase protein kinase. Science **257:**1958–1961.
7. **Eick, D.** 1990. Elongation and maturation of c-myc RNA is inhibited by differentiation inducing agents in HL60 cells. Nucleic Acids Res. **18:**1199–1205.

7a. **Hatzigeorgiou, Artemis.** Submitted for publication.

8. **Henglein, B., X. Chenivesse, J. Wang, D. Eick, and C. Brechot.** 1994. Structure and cell cycle regulated transcription of the human cyclin A gene. Proc. Natl. Acad. Sci. USA **91:**5490–5494.
9. **Hsiao, K. M., S. L. McMahon, and P. J. Farnham.** 1994. Multiple DNA elements are required for the growth regulation of the mouse E2F1 promoter. Genes Dev. **8:**1526–1537.
10. **Jansen-Dürr, P., A. Meichle, P. Steiner, M. Pagano, K. Finke, J. Botz, J. Weßbecher, G. Draetta, and M. Eilers.** 1993. Differential modulation of cyclin gene expression by MYC. Proc. Natl. Acad. Sci. USA **90:**2547–2552.
11. **Johnson, D. G., K. Ohtani, and J. R. Nevins.** 1994. Autoregulatory control of E2F1 expression in response to positive and negative regulators of cell cycle progression. Genes Dev. **8:**1514–1525.
12. **Knoblich, J. A., K. Sauer, L. Jones, H. Richardson, R. Saint, and C. F. Lehner.** 1994. Cyclin E controls S phase progression and its down-regulation during Drosophila embryogenesis is required for the arrest of cell proliferation. Cell **77:**107–120.
13. **Koff, A., F. Cross, A. Fisher, J. Schumacher, K. Leguellec, M. Philippe, and J. M. Roberts.** 1991. Human cyclin E, a new cyclin that interacts with two members of the cdc2 gene family. Cell **66:**1217–1228.
14. **Koff, A., A. Giordano, D. Desai, K. Yamashita, J. W. Harper, S. Elledge, T. Nishimoto, D. O. Morgan, B. R. Franza, and J. M. Roberts.** 1992. Formation and activation of a cyclin E-cdk2 complex during the G1 phase of the human cell cycle. Science **257:**1689–1694.
15. **Lam, E. W. F., and N. B. La Thangue.** 1994. DP and E2F proteins: coordinating transcription with cell cycle progression. Curr. Opin. Cell Biol. **6:**859–866.
16. **Lam, E. W. F., J. D. H. Morris, R. Davies, T. Crook, R. J. Watson, and K. H. Vousden.** 1994. Hpv16 E7 oncoprotein deregulates B-Myb expression—correlation with targeting of P107/E2F complexes. EMBO J. **13:**871–878.
17. **La Thangue, N. B.** 1994. Drtf1/E2F—an expanding family of heterodimeric transcription factors implicated in cell-cycle control. Trends Biochem. Sci. **19:**108–114.
18. **Motokura, T., T. Bloom, H. G. Kim, H. Jüppner, J. V. Ruderman, H. M. Kronenberg, and A. Arnold.** 1991. A novel cyclin encoded by a bc/1-linked candidate oncogene. Nature (London) **350:**512–515.
19. **Nevins, J. R.** 1993. Transcriptional activation by the adenovirus E1A proteins. Semin. Virol. **4:**25–31.
20. **Nordeen, S. K.** 1988. Luciferase reporter gene vectors for analysis of promoters and enhancers. BioTechniques **6:**454–458.
21. **Ohtani, K., J. Degregori, and J. R. Nevins.** 1995. Regulation of the cyclin E gene by transcription factor E2F1. Proc. Natl. Acad. Sci. USA **92:**12146–12150.
22. **Ohtsubo, M., A. M. Theodoras, J. Schumacher, J. M. Roberts, and M. Pagano.** 1995. Human cyclin E, a nuclear protein essential for the G_1-to-S phase transition. Mol. Cell. Biol. **15:**2612–2624.
23. **Pagano, M., G. Draetta, and P. Jansen-Dürr.** 1992. Association of cdk2 kinase with the transcription factor E2F during S phase. Science **255:**1144–1147.
24. **Pagano, M., R. Pepperkok, F. Verde, W. Ansorge, and G. Draetta.** 1992. Cyclin A is required at two points in the human cell cycle. EMBO J. **11:**961–971.
25. **Resnitzky, D., M. Gossen, H. Bujard, and S. I. Reed.** 1994. Acceleration of the G_1/S phase transition by expression of cyclins D1 and E with an inducible system. Mol. Cell. Biol. **14:**1669–1679.
26. **Schulze, A., K. Zerfaß, D. Spitkovsky, J. Berges, S. Middendorp, P. Jansen-Dürr, and B. Henglein.** 1995. Cell cycle regulation of cyclin A gene transcription is mediated by a variant E2F binding site. Proc. Natl. Acad. Sci. USA **92:**11264–11268.
27. **Schwarz, J. K., S. H. Devoto, E. J. Smith, S. P. Chellapan, L. Jakoi, and J. R. Nevins.** 1993. Interactions of the p107 and Rb proteins with E2F during the cell proliferation response. EMBO J. **12:**1013–1020.
28. **Sherr, C. J.** 1994. G1 phase progression: cycling on cue. Cell **79:**551–555.

28a. **Spitkovsky, D.** Unpublished data.

29. **Spitkovsky, D., P. Steiner, J. Lukas, E. Lees, M. Pagano, A. Schulze, S. Joswig, D. Picard, M. Tommasino, M. Eilers, and P. Jansen-Dürr.** 1994. Modulation of cyclin gene expression by adenovirus E1A in a cell line with E1A-dependent conditional proliferation. J. Virol. **68:**2206–2214.
30. **Tommasi, S., and G. P. Pfeifer.** 1995. In vivo structure of the human *cdc2* promoter: release of a p130–E2F-4 complex from sequences immediately upstream of the transcription initiation site coincides with induction of *cdc2* expression. Mol. Cell. Biol. **15:**6901–6913.
31. **Wimmel, A., F. Lucibello, A. Sewing, S. Adolph, and R. Müller.** 1994. Inducible acceleration of G(1) progression through tetracycline-regulated expression of human cyclin E. Oncogene **9:**995–997.
32. **Zerfass, K., A. Schulze, D. Spitkovsky, V. Friedman, B. Henglein, and P. Jansen-Dürr.** 1995. Sequential activation of cyclin E and cyclin A gene expression by human papillomavirus type 16 E7 through sequences necessary for transformation. J. Virol. **69:**6389–6399.
33. **Zindy, F., E. Lamas, X. Chenivesse, J. Sobczak, D. Fesquet, J. Wang, B. Henglein, and C. Brechot.** 1991. Cyclin A is required in S phase in normal epithelial cells. Biochem. Biophys. Res. Commun. **182:**1144–1154.
34. **Zwicker, J., F. C. Lucibello, L. A. Wolfraim, C. Gross, M. Truss, K. Engeland, and R. Müller.** 1995. Cell cycle regulation of the cyclin A, cdc25C and cdc2 genes is based on a common mechanism of transcriptional repression. EMBO J. **14:**4514–4522.