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The G_1/S Boundary-Specific Enhancer of the Rat *cdc2* Promoter

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Multiple species of G_1 cyclins and cyclin-dependent kinases are induced sequentially during G_1 phase, and the expression of cyclin A and *cdc2* genes is subsequently induced at the G_1/S boundary. To analyze the mechanism of *cdc2* promoter activation, the 5′-flanking region of the rat *cdc2* gene was isolated and its structural features were characterized. The highly conserved sequence between human and rat *cdc2* genes is present in the basal promoter region from positions −183 to −122, which contains the E box, Sp1, and E2F motifs. The expression of 5′ sequential deletion derivatives of the promoter fused to luciferase cDNA in rat 3Y1 cells revealed the presence of the enhancer element. The presumed enhancer region was further analyzed by the introduction of base substitutions and by the formation of DNA-protein complexes with cell extracts prepared at various times during the G_1-to-S-phase progression. These analyses revealed that the enhancer sequence, AAGTTACAAATA, located from −276 to −265, confers strong inducibility on the basal promoter at the G_1/S boundary. The base substitutions introduced into the motifs of transcription factors indicated that the E2F motif is essential for the enhancer-dependent activation of the *cdc2* promoter at the G_1/S boundary. Electrophoretic mobility shift assays and DNase I footprinting showed that a factor which interacts with the enhancer element is induced late in G_1 phase.

Cell cycle progression has been shown to be regulated by sequential expression and activation of a variety of protein kinases consisting of a catalytic subunit and a regulatory subunit, called cyclin (51). In growing mammalian cells, the expression of cyclins D and E is induced during G_1 phase, expression of cyclin A is induced at the G_1/S boundary, and expression of cyclin B begins in G_2 phase. Multiple species of cyclin-dependent kinases are also expressed sequentially during G_1 phase and are activated by association with cyclin and phosphorylation in a stage-specific manner. The expression of cdc2 begins concomitantly with that of cyclin A at the G_1/S boundary and reaches a maximal level during S phase (25, 43, 53).

The *cdc2* gene was first identified in fission yeast *Schizosaccharomyces pombe* and encodes a 34-kDa cyclin-dependent kinase (42). The *CDC28* gene in budding yeast *Saccharomyces cerevisiae* is the counterpart of the *cdc2* gene (39). The function of $p34^{cdc2/CDC28}$ kinase is required for both the G_1-S and G_2-M transitions of the cell cycle in yeasts (14). A human homolog of the *cdc2* gene was isolated by complementation in yeasts (32). Human cdc2 is differentially regulated through association with cyclin A and cyclin B (19, 46). Although cyclin A is essential for S-phase entry (20, 56), the cyclin A-cdc2 kinase is activated only in G_2 phase (44). cdc2 is maximally activated at the end of G_2 phase by association with cyclin B, triggering the entry into M phase (6, 46). In addition to these findings, several experiments suggest that $p34^{cdc2}$ is required only for the G_2-M transition and may not be involved in the G_1-S transition in mammalian cells. Inhibition of the $p34^{cdc2}$ kinase activity by microinjection of anti-cdc2 antibody prevents cell division without affecting DNA synthesis (47). A mouse cell line carrying the temperature-sensitive mutation in the *cdc2* gene is unable to enter M phase at the restrictive temperature, while DNA synthesis remains normal (23, 52). The G_1-S transition in mammalian cells seems to be primarily regulated by cdk2, which was isolated as a member of the cdc2 family and can rescue the *cdc2*/*CDC28* gene defect in yeasts (11, 41, 54). Transcription of the *cdk2* gene increases before that of *cdc2* in growth-stimulated cells (10, 25, 41). Depletion of the endogenous cdk2 in *Xenopus* extracts blocks DNA replication (13), and the cyclin A-cdk2 complex has been shown to associate with the initiation complex of DNA replication (15). Activation of the cyclin A-cdk2 kinase occurs concomitantly with the phosphorylation of the RP-A replication factor at the G_1/S boundary (8, 9, 48). In contrast, the involvement of cdc2 in the G_1-S transition has been reported for T lymphocytes (16) and Burkitt's lymphoma cells (34).

Although cdc2 lacks a clear role for the G_1-S transition, expression of cdc2 is induced at the G_1/S boundary (25, 43, 53). Analysis of the human *cdc2* promoter also showed that its activity is negatively regulated during G_1 phase, presumably by pRB, that it is released from the negative regulation at the G_1/S boundary (4). pRB and its related proteins bind to transcription factors, such as the E2F-DP1 heterodimer, and inhibit their activities (40). Phosphorylation of pRB by cyclin D-cdk4 (5, 12, 27, 35) and cyclin E-cdk2 (7, 29) during middle to late G_1 phase results in the loss of ability to bind to these factors, thereby releasing the active form of free transcription factors. The E2F recognition sequence has been found in the promoters of various late-response genes, e.g., those encoding dihydrofolate reductase (1), DNA polymerase α (45), and cdc2 (4). These promoters are activated shortly before or simultaneously to S-phase entry in accordance with the previous observation that the E2F-specific complexes are formed during this stage (25). An essential role of E2F for S-phase entry has

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been shown by the induction of S-phase entry in quiescent cells after microinjection of E2F-1 (26).

In this study, the 5′-flanking region of the rat *cdc2* gene was isolated and its structural features were analyzed to identify the promoter element which confers inducibility at the G_1/S boundary. Analysis of the 5′ sequential deletion derivatives of the promoter and introduction of base substitutions into the presumed promoter elements revealed the enhancer element that is specifically activated at the G_1/S boundary. The E2F motif is essential for this enhancer-dependent activation of the *cdc2* promoter. A factor which interacts with this enhancer element seems to be induced in late G_1 phase.

MATERIALS AND METHODS

Cell lines. The 3Y1-B cell line, clone 1-6, is a clonal line of Fischer rat embryo fibroblasts (28). A 3Y1 derivative cell line, g12-21, was established by introducing 3Y1 cells with pM12SG in which the adenovirus type 2 E1A 12S ($E1A_{12S}$) cDNA was placed downstream of the mouse mammary tumor virus long terminal repeat (25). The cell line expresses the 12S cDNA efficiently in response to dexamethasone (DEX). Both 3Y1 and g12-21 cells were cultivated at 37°C in Dulbecco's modified Eagle's medium with 10% fetal calf serum (FCS).

Cloning and DNA sequencing of rat *cdc2* promoter region. A rat genomic DNA library was constructed by cloning the *Sau*3AI partially digested normal rat kidney genomic DNA fragments with lengths of 10 to 20 kb to the λDASH II vector at the *Bam*HI site. The library was screened for the 5′-flanking region of the *cdc2* gene with ^{32}P-labeled rat *cdc2* cDNA probes of the *Hin*dIII-*Sal*I fragment (between positions +17 and +380) and the *Pst*I fragment (between positions +98 and +477). Three clones containing the same sequence were isolated. One clone was cleaved with *Not*I, and the 9-kb fragment containing the 5′-end portion of the *cdc2* cDNA was cloned at the *Not*I site of pBluescript II KS. The cloned fragment was further cleaved with *Pst*I, and the 2.5-kb fragment containing the 5′-flanking region was recloned at the *Pst*I site of pBluescript II KS to generate pN2BN9 P. Both strands of the 2.5-kb fragment were sequenced by the dideoxy chain termination method (50).

Construction of *cdc2* promoter-CAT and *cdc2* promoter-luciferase fusion plasmids. To shorten the length of cDNA contained in pN2BN9 P, the *Kpn*I and *Sal*I sites in the multicloning site were cleaved, and the synthetic oligonucleotide containing the Δ*Kpn*I, *Bgl*II, *Kpn*I, and *Sal*I sites was inserted after cleavage with *Kpn*I and *Sal*I to generate pN2BN9 PB (see Fig. 2A). pN2BN9 PB was then cleaved with *Kpn*I and *Sal*I and digested with Exo III, which acts only on the 3′ end of the break with single-strand extension at the 5′ end (*Sal*I site) (24, 55). The resulting single-stranded DNA was digested with mung bean nuclease. The DNA was then circularized by blunt-end ligation. The cDNA was shortened up to position +64. pN2BN9 PB was then cleaved with *Bgl*II and *Pst*I, and the fragment containing the 5′-flanking region was inserted at the *Bam*HI-*Pst*I site of pBluescript II KS containing the *Bgl*II site adjacent to the *Kpn*I site in the reverse direction to generate pN2BN9 PB-R (see Fig. 2B). The 5′ sequential deletion derivatives of the 5′-flanking region was similarly constructed after cleavage of pN2BN9 PB-R with *Kpn*I and *Sal*I. For the construction of various *cdc2* promoter-chloramphenicol acetyltransferase (CAT) constructs, the *Hin*dIII site of pSV2CAT-XB (38) was converted to the *Xba*I site to generate pSV2CAT-XbaI (see Fig. 2C). The 5′ deletion derivatives of the 5′-flanking region in pN2BN9 PB-R was then isolated by cleavage with *Xba*I and *Bgl*II and inserted at the *Xba*I-*Bgl*II site of pSV2CAT-XbaI. The *cdc2* promoter-luciferase constructs were similarly constructed. The *Xba*I-*Bgl*II fragments containing the 5′ deletion derivatives of the 5′-flanking region were inserted upstream of the luciferase cDNA at the *Kpn*I-*Nhe*I site of PGV-B (Nippongene) by using the *Bgl*II-*Kpn*I adaptor (5′-GATCGTAC-3′). The cohesive ends of *Xba*I and *Nhe*I share the same sequence (5′-CTAG-3′).

Substitutive mutations at E box, Sp1, and E2F motifs and enhancer element. pN2BN9 PB-R31 containing the *cdc2* promoter region between −310 and +64 (for reference, see Fig. 2B) was cleaved with *Pvu*II and *Xba*I, and the fragment containing the promoter region was inserted at the *Sma*I-*Xba*I site of M13mp18 to generate the M13 cdc2-31 recombinant phage. Substitutive mutations at the E box, Sp1, and E2F motifs or the enhancer element were created by site-specific mutagenesis as described by Kunkel et al. (31) (see Fig. 5 and 7). These substitutions were confirmed by DNA sequencing. The double-stranded M13 cdc2-31 recombinant phage DNA carrying the base substitution was recovered and cleaved with *Xba*I and *Bgl*II, and the *Xba*I-*Bgl*II fragment containing the mutated *cdc2* promoter was inserted at the *Kpn*I-*Nhe*I site of PGV-B to generate pcdc2luc31 carrying the substitutive mutation as described above. The double mutant of pcdc2luc31 carrying the substitutions at both the E2F motif and the enhancer element (see Fig. 10A) was constructed by insertion of the substitutive mutation at the enhancer element into pcdc2luc31 carrying the substitution at the E2F motif.

End labeling of DNA fragments and oligonucleotides. DNA fragments were prepared by PCR (49). PCR was performed by using a thermal reactor (Hybrid) with Ampli*Taq* DNA polymerase (Perkin-Elmer Cetus). The PCR products were purified by agarose gel electrophoresis and labeled at 5′-hydroxyl termini with T4 polynucleotide kinase in the presence of [γ-^{32}P]ATP. The labeled fragment was cleaved with an appropriate restriction enzyme to generate probe DNA in which only one strand was end labeled.

Oligonucleotides were synthesized on an Applied Biosystems DNA synthesizer. All oligonucleotides were purified by an OPC cartridge column (Applied Biosystems). For electrophoretic mobility shift assays, purified single-stranded oligonucleotides were phosphorylated with T4 polynucleotide kinase in the presence of [γ-^{32}P]ATP. Oligomers were annealed in 10 mM Tris hydrochloride (pH 7.5) containing 1 mM EDTA, 200 mM NaCl, and 5 mM $MgCl_2$. The remaining single-stranded probe was removed by acrylamide gel electrophoresis, and the double-stranded probe was isolated and stored in 10 mM Tris hydrochloride (pH 7.5) containing 1 mM EDTA and 200 mM NaCl at −20°C.

Transient transfection and analysis of gene expression. DNA transfection was performed by using the $CaPO_4$ coprecipitation procedure (22) as modified by Chen and Okayama (3). Growing rat 3Y1 cells were transfected with 20 μg each of the *cdc2* promoter-CAT constructs, and CAT activities were assayed at 48 h after transfection according to the method of Gorman et al. (21). Protein (120 μg) from the cell extract was used for each assay. For analysis of *cdc2* promoter activity during the cell cycle progression, confluent monolayers of g12-21 cells, a derivative of 3Y1 cells, were transfected with 20 μg each of the *cdc2* promoter-luciferase construct and maintained in low-serum (0.5% FCS) medium for 48 h. Cell growth was then stimulated by either replacing the medium with fresh medium containing 10% FCS or by the addition of 10^{-6} M DEX to induce the $E1A_{12S}$ expression. The cells harvested at various intervals were assayed for luciferase activity with 120 μg of protein from the cell extract and 100 μl of the luciferin substrate (Nippongene), with a LB9501 luminometer (Berthold) (37).

Preparations of whole-cell extract. Whole-cell extracts were prepared essentially according to the method of Manley et al. (33). g12-21 cells were washed in phosphate-buffered saline containing 0.5 mM $MgCl_2$ and suspended in 4 volumes of hypotonic buffer (10 mM Tris hydrochloride [pH 7.9 at 4°C], 1 mM EDTA, 5 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride [PMSF]). After 20 min, the cells were homogenized, and 4 volumes of sucrose-glycerol solution (50 mM Tris hydrochloride [pH 7.9 at 4°C], 10 mM $MgCl_2$, 25% [wt/vol] sucrose, 50% [vol/vol] glycerol, 2 mM dithiothreitol, 0.5 mM PMSF) was added. After gentle stirring, 1 volume of saturated $(NH_4)_2SO_4$ was added dropwise, and the homogenate was centrifuged at 53,000 rpm at 4°C for 3 h in a Hitachi RP65T rotor. Solid $(NH_4)_2SO_4$ to a final concentration of 0.33 g/ml was added to the supernatant, and the supernatant was centrifuged (19,200 rpm) in a Hitachi RP65T rotor for 30 min. The precipitate was dissolved in a minimal volume of D buffer (20 mM HEPES [*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid, pH 7.9], 12.5 mM $MgCl_2$, 0.1 mM EDTA, 20% [vol/vol] glycerol, 2 mM dithiothreitol, 0.5 mM PMSF) containing 0.1 M KCl. The sample was dialyzed against two changes of 1 liter each of D buffer containing 0.1 M KCl for 1 h and centrifuged at 15,000 rpm for 15 min. The supernatant was quickly frozen in dry ice-ethanol and stored at −80°C.

Electrophoretic mobility shift assays. DNA-protein complexes were formed in a 10-μl reaction mixture (36) containing 20 mM HEPES buffer (pH 7.9), 100 mM KCl, 12.5 mM $MgCl_2$, 1 mM EDTA, 20% [vol/vol] glycerol, 2 mM dithiothreitol, 0.5 mM PMSF, 1 μg of poly(dI-dC) · poly(dI-dC), 0.5 fmol (approximately 5×10^3 cpm) of ^{32}P-labeled oligonucleotide, and whole g12-21 cell extract after incubation at 0°C for 30 min. Competitor DNA was added at the same time as probe DNA. DNA-protein complexes were resolved by electrophoresis on 5% polyacrylamide gels at 4°C for 2.5 h at 250 V in TGE buffer (25 mM Tris hydrochloride [pH 8.0], 192 mM glycine, 2 mM EDTA). The gels were dried and autoradiographed with an intensifying screen at −80°C.

DNase I footprinting. Footprinting analysis was performed as described by Galas and Schmitz (18) and Matuno et al. (36). The extract (15 mg of protein) prepared from g12-21 cells after serum stimulation for 12 h was applied onto a 2-ml heparin-Sepharose CL-6B column (Pharmacia LKB Biotechnology) equilibrated with D buffer containing 0.1 M KCl. The proteins were eluted successively with D buffer containing 0.1, 0.2, 0.4, 0.8, and 1.2 M KCl. A DNA-protein complex was formed in 60 μl of the reaction mixture, which contains 5 to 10 μg of protein, 0.8 M KCl elute, 1 μg of poly(dI-dC) · poly(dI-dC), 10 μg of *Escherichia coli* tRNA, and 0.2 fmol (approximately 10^4 cpm) of the ^{32}P-labeled oligonucleotide containing the promoter sequence between −314 and −184. After incubation at 0°C for 30 min, 2 to 6 μg of DNase I was added, and the solution was incubated at 0°C for 1 min. The reaction was terminated by the addition of 60 μl of 20 mM EDTA containing 1% sodium dodecyl sulfate, 0.2 M NaCl, and 20 μg of proteinase K, and the solution was incubated at 42°C for 1 h. After extraction with phenol-chloroform and precipitation with ethanol, DNA was electrophoresed on 8% polyacrylamide gels containing 7 M urea.

RESULTS

Structural features of the 5′-flanking sequence of the rat *cdc2* gene. The 5′-flanking region of the rat *cdc2* gene was isolated from a rat genomic DNA library prepared from normal rat kidney cells by using the λDASH II cloning vector. One of the three positive clones isolated by using the 5′-end portion

of the rat *cdc2* cDNA as a probe was cleaved with *Not*I, and the 9-kb fragment was recloned into pBluescript II KS. The recombinant plasmid was further cleaved with *Pst*I, and the 2.5-kb fragment containing the 2.2-kb 5′-flanking sequence and the 0.3-kb downstream sequence including the 5′ portion of the cDNA was recloned into pBluescript II KS to generate pN2BN9 P. The nucleotide sequence determined by the dideoxy chain termination method is shown in Fig. 1A. The consensus binding sites for known transcription factors were determined by computer analysis. The 5′-flanking sequence from −700 to −1 has 60% homology with that of the human *cdc2* gene. The proximal region between −224 and −122, which contains the ATF-like sequence and the E box, Sp1, and E2F recognition sequences, has an especially high degree of homology with the corresponding region of the human *cdc2* promoter, as shown in Fig. 1B. Upstream in this highly conserved region, there are three PEA3, two C/EBP, and one E2A motifs. These motifs are also present in the corresponding upstream region of the human *cdc2* promoter, although the positions and numbers of these motifs are different for the rat and human promoters.

***cdc2* promoter elements concerned with activation at the G_1/S boundary.** To analyze the elements that are involved in the induction of the rat *cdc2* promoter activity at the G_1/S boundary, 5′ sequential deletion derivatives of the promoter were fused to an *E. coli* CAT gene, as shown in Fig. 2 and 3. These *cdc2* promoter-CAT constructs (pcdc2CAT) were transfected to growing rat 3Y1 cells, and CAT activities were measured 48 h posttransfection. The transfection was repeated three times, and average values are presented in Fig. 3. The activity expressed by pcdc2CAT219, which contains the 5′-flanking sequence up to −2193, was taken as 1. CAT activity increased by more than 10-fold when the promoter sequence was deleted up to −310. Since the activity of pcdc2CAT31 was consistently higher than that of pcdc2CAT58 by nearly 10-fold, the sequence between −589 and −310 seemed to contain a negative element for expression of the *cdc2* promoter. Further deletion up to −195 resulted in a drastic decrease in the promoter activity to less than 10-fold, suggesting the presence of an enhancer element in the sequence between −310 and −195. All of the other constructs showed activities less than 1/10 of that expressed by pcdc2CAT31.

To see the involvement of this enhancer element in the activation of the *cdc2* promoter at the G_1/S boundary, recombinant plasmids containing luciferase cDNA downstream of the 5′ sequential deletion derivatives of the promoter were constructed (pcdc2luc). Luciferase is known to have a much shorter half-life than does CAT, so that the promoter activity at each stage of cell cycle progression can be estimated more accurately. These luciferase constructs were transfected to confluent monolayers of g12-21 cells that were established from 3Y1 cells after introduction of adenovirus E1A 12S cDNA linked to the hormone-inducible promoter (25). Expression of $E1A_{12S}$ can be induced in g12-21 cells by the addition of 10^{-6} M DEX to the culture medium. The transfected cells were maintained in low-serum (0.5% FCS) medium for 48 h to synchronize the cells in the G_0 state. The growth was then stimulated by either the addition of fresh medium containing 10% FCS or by the addition of DEX to induce E1A expression. Luciferase activity was assayed at various times after growth stimulation. The transfection experiments were repeated twice, and average values were plotted (Fig. 4). Luciferase activities of all the constructs were induced at the G_1/S boundary in both the serum-stimulated (Fig. 4A) and the DEX-treated (Fig. 4B) cells. With both treatments, a marked induction was observed with pcdc2luc31 in cells at the G_1/S boundary when the rate of [^{3}H]thymidine uptake into the acid-insoluble fraction began to increase (data not shown). The luciferase activity increased extensively during S phase. The induction occurred earlier in the DEX-treated cells, since $E1A_{12S}$ shortened the G_1 phase, as previously shown (25). The level of activity induced by pcdc2luc11, which lacks the highly conserved sequence containing the E box, Sp1, and E2F motifs, was very low. The levels of activities induced by pcdc2luc219, pcdc2luc58, and pcdc2luc19 were intermediate between those induced by pcdc2luc31 and pcdc2luc11 in serum-stimulated cells (Fig. 4A). In DEX-treated cells (Fig. 4B), the levels of activities induced by pcdc2luc219 and pcdc2luc58 were also intermediate, but the level of activity induced by pcdc2luc19 was very low. The reason for this low level of activity is presently unclear. These results indicate that the enhancer element estimated from the promoter activities of various pcdc2CAT constructs in growing 3Y1 cells (Fig. 3) is involved in the induction of the *cdc2* promoter activity at the G_1/S boundary. The extents of the promoter activity induced by pcdc2luc19 and pcdc2luc58 were consistently lower than that induced by pcdc2luc31, suggesting that the negative element resides in the upstream of the enhancer is not completely suppressed at the G_1/S boundary and in S phase. A small but significant induction of the promoter activity by pcdc2luc19 lacking the enhancer element suggests that the activation of the *cdc2* promoter is also regulated by some factors other than the enhancer element.

Involvement of E2F motif in enhancer-dependent activation of the *cdc2* promoter at the G_1/S boundary. To analyze the requirement of the E box, Sp1, and E2F motifs located in the highly conserved region of the basal *cdc2* promoter for the enhancer-dependent activation of the promoter at the G_1/S boundary, 2- or 3-base substitutions were introduced into each motif of pcdc2luc31 by site-directed mutagenesis, as described in the legend to Fig. 5. These luciferase constructs carrying the substitutive mutation were transfected to confluent monolayers of g12-21 cells, and luciferase activities were similarly assayed after growth stimulation by serum (Fig. 5). Introduction of the substitution into the E box had no effect on the promoter activity, and the pattern of luciferase activity induced by pcdc2luc31 carrying the substituted E box was the same as that induced by pcdc2luc31. In contrast, the induction of luciferase activity by pcdc2luc31 carrying the substitution in the E2F motif was delayed to mid-S phase, and the extent of induction was much lowered, indicating that the E2F motif plays an important role in the induction of the *cdc2* promoter activity at the G_1/S boundary and in S phase. Introduction of the substitution into the Sp1 motif also had a significant effect on the induction of the promoter activity, and the induction of luciferase activity by pcdc2luc31 carrying the substituted Sp1 motif was delayed to early S phase.

Analysis of DNA-protein complexes formed at the presumed enhancer region during cell cycle progression. To analyze the DNA-protein complexes formed at the enhancer element, the sequence between −310 and −195 was divided into four portions, and oligonucleotides I, II, III, and IV, each about 40 bp in size, were chemically synthesized (Fig. 6). These fragments contain overlapping sequences with lengths of about 10 bp at both ends with adjacent fragments so that the entire sequence could be analyzed for protein interaction. The ^{32}P end-labeled oligonucleotide was incubated with extracts prepared from quiescent g12-21 cells after serum stimulation for 0, 11, 14, and 20 h. These stages correspond to the G_0 state, late G_1 phase, the G_1/S boundary, and S phase, respectively. The complexes formed in the presence and absence of an excess of the same unlabeled oligonucleotide were analyzed by electrophoretic mobility shift assays. The complexes were formed with these

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-2193        TCA AGCCAAATGG AGACCCATCA TGGAGAGGGG AAGCAGACAT GGGCTCCCGC TCCTAACCAA GAAGCTACCT GCAACTGATG CCAGAGTCGC
-2100 CAAGGGAAAG TCAGTTTCTC CAAGGCAGTG CAACCCGGTT TATCAACTGT ACTTCAGGGT CTGCATAATG CCCAGGAGTA GCTGGCCAAC ACAAAATGAA
-2000 CTCCATGGTC TTTTTATGGA CTTTTGTTTG ATTTTGCTTT GTTTTGTCTC TGTTGTTCTA ACTAGTCTTT TGCTTGTTTT GATTTTGGTT TTCTGGGTTT
-1900 TGTTTGTCTG TTTCTTGTTC TGTTGTTTTG GGAGAGTGAG AGACAATCCC CTCTCCCCCC AAAACACACA CCCACACACC CACACAGGGA GACAAAAAAA
-1800 GAGAGAGGCA GACAAACAAG ACACACAGAG ACAGAGAGAA AGAGAAAGAG ACACACAGAT ATAGACAGAC ACATACACTG TTTGCTTTCT AAGGGATATT
-1700 TAACATTTCT TTTTTAAGAT TTATTTATTT AGGATACAGT AGTCTACCTG CACGTGTGCC TGCACACCAG AAGAGGGCAC CAGATCCCAT TTTAGATGGT
-1600 TATGGACCAT CATGTGGTGG CTGGGATTTG AACTCAGGAC CTCTGGAAGA GCAGTCAGTG CTCTTAACCA CTGAGCCATC TCTCCAGCCC AGTATTTAAC
-1500 ATTCTTTTGT GGAGATATGT CCAAGCATTT AATGTGTGTA CCATTTTATA CATAATGTAA AACCAAAGCA GGGATACATA AGTGTGAAAT CACAGATAGG
-1400 CGGGCATTTT CTCTTTTCAG TTTCCAGAGT GTCTTTCAGG GTTTCATTGG TTCCTTTGCC CTTGTCTTCT CCAGACACTT TAAATGTGAT CCTTCTGCTG
-1300 GCCACTAATC ACTCATTAGC AAAACTGTAA TCATGGAACA TGGGGCCATC TGGGAAATAC ATCAAGGGAG CTAACAGAGA CATACTGAGA CAGCGACTTC
                                                                                          PEA3
-1200 ATGTTCCTTT AAATATGCGG ATCCTAGCAT GTAGTGGTCA TATGTTAGTG TGTATATAAG CTTTGAACTA CAAAGGAAGG GTCACCAGAA GGGAAAAATG
-1100 AGAGTCATGG ACATGGTGCC AGGACATGGG AGGCTATGGG GGTGGGGGTG GGCAGTTACA AGATGGGGGT ATTAGGGGGC TGGGGATTTA GCTCAGTGGT
                  PEA3
-1000 AGAGCGCTTA CCTAGGAAGC GCAAGGCCCT GGGTTCGGTC CCCAGCTCCG AAAAAAAAAA GAACCAAAAA AAAAAAAGAT GGGGGTATTA GGGTTACTGG
                                C/EBP                                                                      E2A
 -900 AGAAGGATGA GAAGAATTAA CACAGACCAA TAACATTTCA CAATTCCATA GTGAAACTTA ATATTATGTA TGTATATATT ATAGCTATAT ATAAAAACAG
 -800 ATGTTTATAT ACACAAACAT ACACACATGT GCACATACAT ATGCCAGTGC ACATACACAC AACATATGCA CACACACACA CATGCCTGCT GCTGTGTGTG
 -700 CTAGGATTAA AGGCATGTGC CACAATCCTC AGCTTAATAT ATATTTTTGA AAGGCATAAT CATAAAGTAA TTTTTCCCCC TTTGGAGCTG AGGACCAAGT
                      C/EBP
 -600 GTTCTATCAC TGGGCTAAAT CTCCAATTCC AGAAAGTAAT TTTAAATGTT AGCAGTAGCC AGGTGGTGGT GGCAGCAGCA CGCACCTTTA ACCTGGGCAC
 -500 TCAGGAGGCA GAAGCATGAT GATATCTGTG AGTTCAAGCC CAGCCTTGTT TACAGAGCCA GTTCCAGGAC AGTCAAGACT ACACAGAGAA AACTTGTCTC
 -400 AAAAAAAGCA AAACCAAACA AAATAAAAGT AGTAAAAATA AGAAACTCTT GATGTAGTGG CATTATCACT TTTGTGGCTA GCTATCTAAA TGCAATACAT
 -300 TTTAATTGTG TTAATCCTGA AAGAAAGTTA CAAATAGAAA TGTAGAGGGA GGGGGGAAAA ACAGATTAGA CTGTCCAGAA ACAACAAGAC GACATTGGAA
      PEA3                    E box      Sp1                                  E2F
 -200 GGAAAGCTGA GCTCAAGAGT CAGTTGGCGC CCGCCCTCCT GTAATTCCTC CGGCCGCGGT TTCCGCTCCC TTTCGCGCTC TGCACTCCCA GGCAGCCCGG
 -100 GCCGTAGCTT AGCTCGGCTC TGATTGGCTC CTTTGAACGT CTACGTGCAA TCGGATTGGC GGACCCGGGG AGCTTTACCG CGGTGAGTTT GAAACTGCTG
   +1 GCACTCGGCC TCTAAGCTCC CGGAGTCGCT GCGTCCGCGC AGTCCGGAAC TGCGGTGTGG CCACCGCGGG AACAGAGAGG TATGGCGTGA GTCGGCAGGG
 +101 TGCCGGCAGG GCCTGCGGGG GCTCCACGGG AGCCGGGGTG TGCCCCGGGG GGGGACAGGC AGGACATCCT GTGAGTCCGG GGGACCCCCT TGTGGACGGG
 +201 TAGAGCCTCC ACGTGAGATC ATGGTC
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B

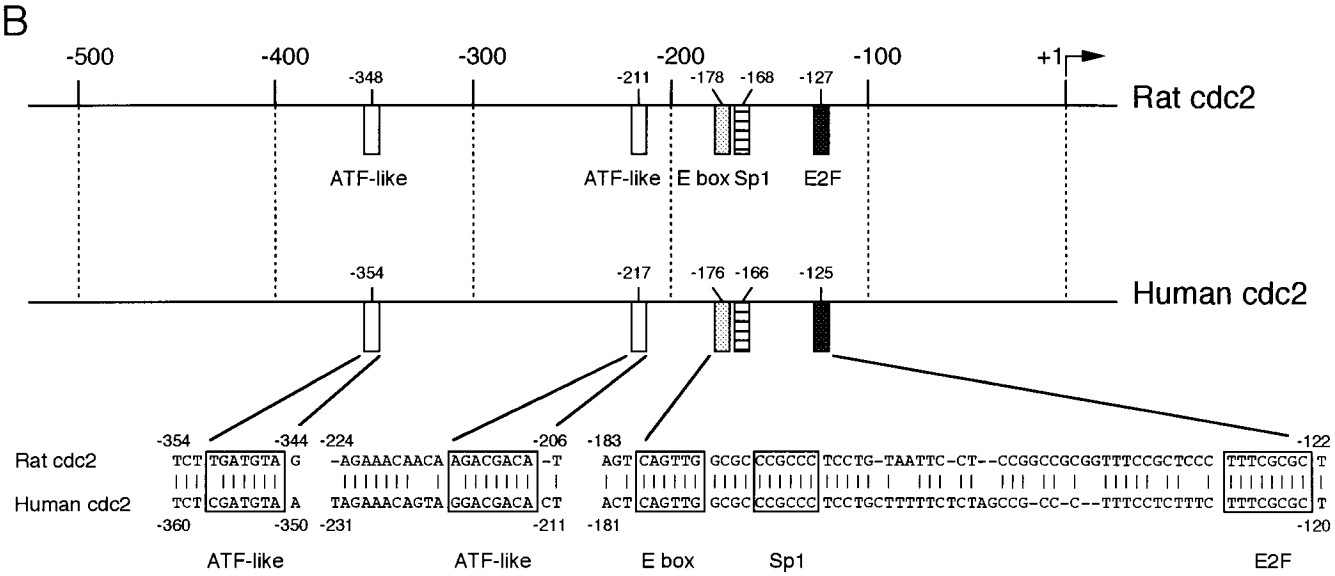

FIG. 1. Structural features of 5′-flanking region of the rat *cdc2* gene. (A) Nucleotide sequence of the 5′-flanking region. The G designated as +1 is the start site of transcription, and the arrow indicates its direction. The first intron is underlined. The positions of consensus sequences for known transcription factors, determined by computer analysis, are boxed. (B) Sequences highly homologous between rat and human *cdc2* promoters. These sequences contain the ATF-like binding sequence, E box, Sp1, and E2F motifs, as shown in boxes.

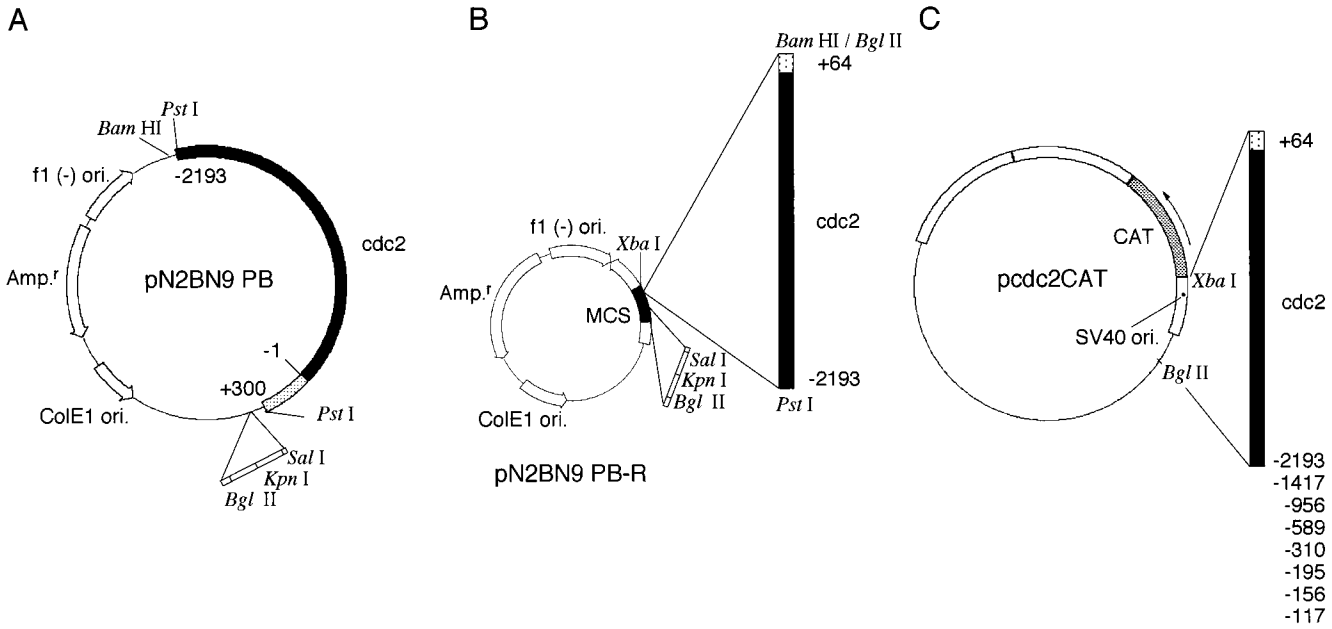

FIG. 2. Structures of recombinant plasmids used for construction of *cdc2* promoter-CAT fusion plasmids. (A) pN2BN9 PB was constructed by insertion of the *cdc2* promoter region from positions −2193 to +300 at the *Pst*I site of pBluescript II KS. The *Bgl*II site was also inserted adjacent to the *Kpn*I site. (B) pN2BN9 PB-R was constructed by insertion of the *cdc2* promoter region from positions −2193 to +64 at the *Bam*HI-*Pst*I site of pBluescript II KS containing the *Bgl*II site in the multicloning site (MCS) in a reverse direction. (C) pcdc2CAT was constructed by insertion of a 5′ sequential deletion derivative of the *cdc2* promoter into the *Xba*I-*Bgl*II site of pSV2CAT-XbaI.

oligonucleotides, but their formations were inhibited by the same unlabeled oligonucleotide to various extents. Among them, the amount of complex formed with oligonucleotide II increased greatly with the cell extracts prepared after late G_1 phase (Fig. 6, panel II, lanes 3 to 5). The formation was abolished almost completely by the unlabeled oligonucleotide (Fig. 6, panel II, lanes 7 to 9). The amount of the complex formed with the 4-h extract (from early G_1 cells) was essentially the same as that formed with the 0-h extract (data not shown). The amount of the complex formed with oligonucleotide IV also increased with the cell extracts prepared after late G_1 phase; however, the amount formed was much less than that formed with oligonucleotide II, and the competition by the unlabeled oligonucleotide was incomplete. Essentially, the same patterns of complex formation were obtained with the cell extracts prepared from DEX-treated g12-21 cells in late G_1 phase, at the G_1/S boundary, and in S phase (data not shown). The complex formation with oligonucleotide I was not efficiently inhibited by the unlabeled oligonucleotide, and the amount of complex formed with oligonucleotide III was unchanged with extracts prepared from the quiescent cells and the cells after late G_1 phase. On the basis of these results, we focused on the complex formed with oligonucleotide II for further analysis of the enhancer element.

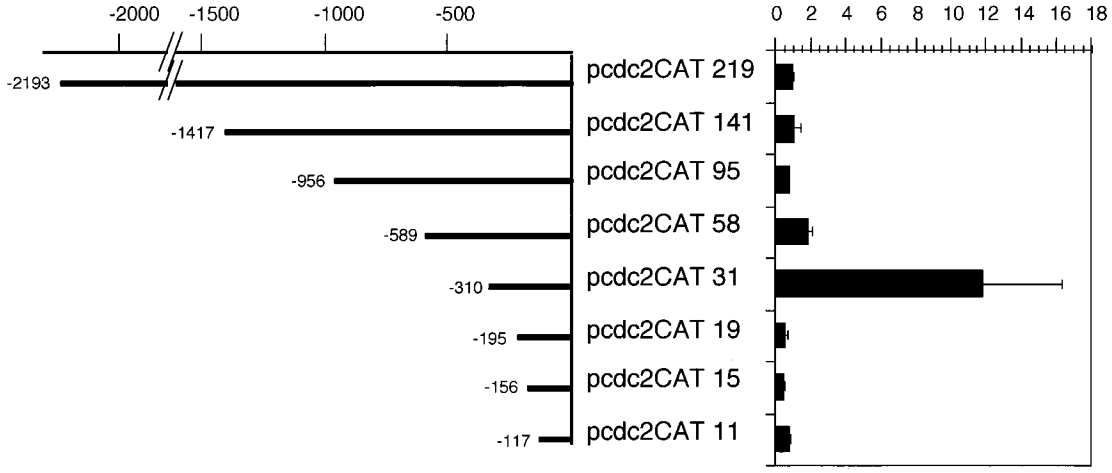

FIG. 3. Analysis of rat *cdc2* promoter elements. The 5′ sequential deletion derivatives of the *cdc2* promoter were fused to the CAT gene. The numbers indicate the 5′-end positions of the promoter sequence. Growing rat 3Y1 cells were transfected with 20 μg each of the CAT constructs, and CAT activities were assayed after 48 h. The values shown are averages for three independent experiments (error bars indicate standard deviations) after quantitation by using a BAS2000 imaging analyzer system. The CAT activity expressed by pcdc2CAT219 was taken as 1.

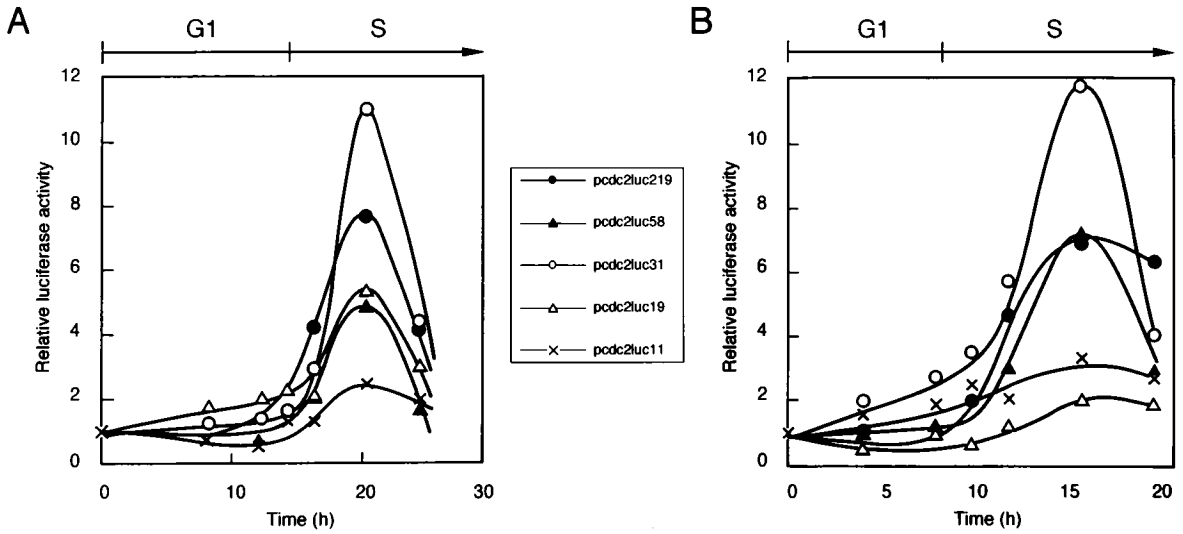

FIG. 4. Rat *cdc2* promoter sequence involved in cell cycle-dependent activation in growth-stimulated cells. Confluent monolayers of g12-21 cells, a derivative of rat 3Y1 cells, were transfected with 20 μg each of the *cdc2* promoter-luciferase constructs and maintained in low-serum (0.5% FCS) medium for 48 h. Cell growth was then stimulated either by replacing the medium with fresh medium containing 10% FCS (A) or by the addition of 10^{-6} M DEX to induce E1A expression (B). The cells were harvested at the times indicated, and luciferase activities were assayed with 120 μg of protein from the cell extracts. The lowest activity expressed by the 0-h extract from unstimulated cells was taken as 1. The values shown are averages for two independent experiments.

Dissection of the presumed enhancer region by introduction of base substitutions. To dissect the enhancer region further, three oligonucleotides, IIa, IIb, and IIc, covering the 5′-end, central, and 3′-end portions of the oligonucleotide II, respectively, were synthesized and used as competitors for complex formation with oligonucleotide II and the extract prepared from quiescent g12-21 cells after serum stimulation for 12 h (Fig. 7A and B). Complex formation was significantly inhibited by competitor IIb to an extent similar to that with unlabeled oligonucleotide II. The presence of competitors IIa and IIc had almost no effect on complex formation. To confirm the interaction of a factor and oligonucleotide IIb, a 3-base substitution was introduced into two sites of competitor IIb, as shown in Fig. 7C. The substituted oligonucleotides, $IIb1^{mut}$ and $IIb2^{mut}$, were used similarly as competitors (Fig. 7D). Competitor $IIb1^{mut}$ lost the ability to inhibit complex formation almost completely, while competitor $IIb2^{mut}$ lost the ability partially. The result indicated that the enhancer element resides in the 5′ side of oligonucleotide IIb and the sequence $^{-272}$TAC^{-270} is essential for binding of a factor to the enhancer element.

To demonstrate that an enhancer-binding factor is abundantly present in the extract prepared from the late G_1 cells, but not in the extract prepared from the quiescent cells, the complex formation was performed with ^{32}P end-labeled oligonucleotide IIb and the 0- and 12-h cell extracts in the presence and absence of the competitors (Fig. 8). The amount of the complex formed with the 0-h extract was much less than that formed with the 12-h extract. The complex formation with the 0-h extract was not completely inhibited by all the competitors. In contrast, complex formation with the 12-h extract was completely inhibited by competitors IIb and II, was moderately inhibited by $IIb2^{mut}$, but was nearly unaffected by $IIb1^{mut}$ and IIa.

The enhancer sequence to which a factor interacts was also analyzed by DNase I footprinting with the ^{32}P end-labeled 130-bp oligonucleotide containing the enhancer region and the 12-h extract. The 12-h extract was applied onto a heparin-Sepharose CL-6B column, and the proteins bound to the column were eluted successively with D buffer containing increas-

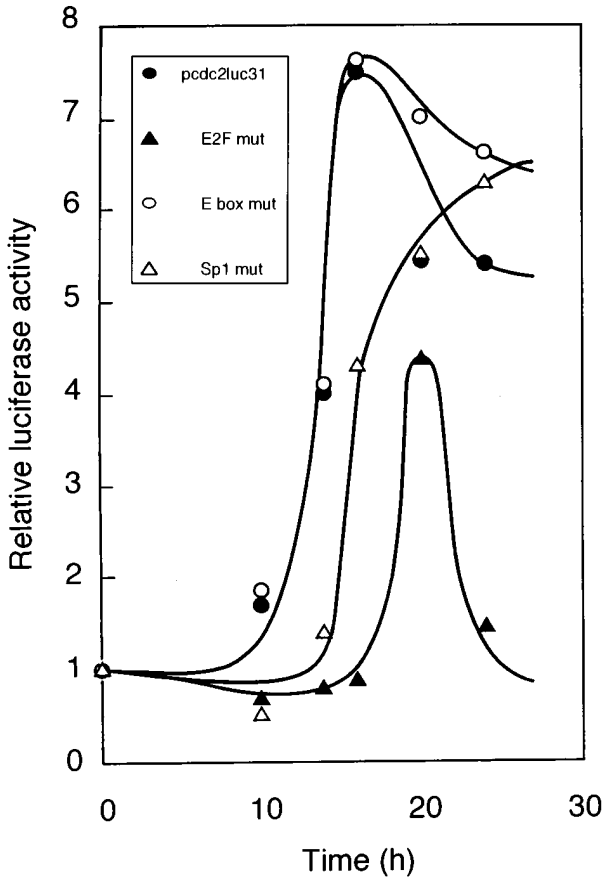

FIG. 5. Involvement of E2F motif in induction of *cdc2* promoter activity at the G_1/S boundary. Confluent monolayers of g12-21 cells were transfected with 20 μg each of pcdc2luc31 carrying the base substitutions in the E box, Sp1, or E2F motif. The cells were made quiescent, and cell growth was stimulated by serum as described in the legend to Fig. 4. The luciferase activities shown are averages for two independent experiments. The base substitutions introduced (underlined) were TTTCGCGC to TTTCGAACC for E2F, GGGCGG to ATT CGG for Sp1, and CAGTTG to CTGCAG for the E box.

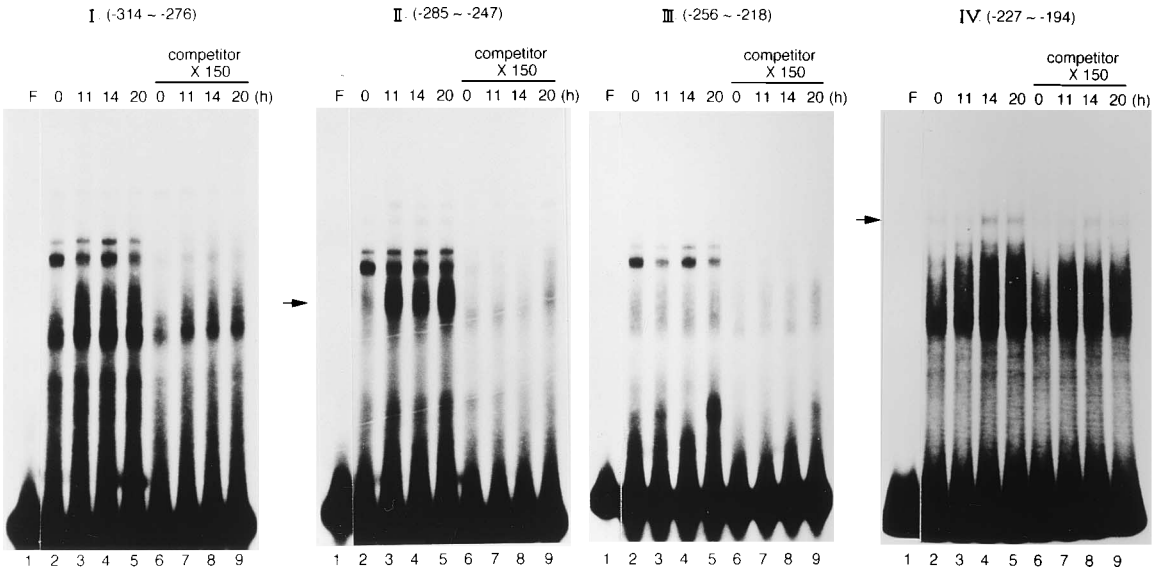

FIG. 6. Electrophoretic mobility shift assays of complexes formed by promoter sequences presumed to contain the enhancer element. Double-stranded oligonucleotides I, II, III, and IV, covering the promoter sequence between −310 and −195, were chemically synthesized and ^{32}P end labeled. Cell extracts were prepared from quiescent g12-21 cells (0 h) and cells in which growth was stimulated by serum for 11 h (late G_1), 14 h (G_1/S boundary), and 20 h (S), as indicated above each lane. The labeled oligonucleotides (0.5 fmol) were incubated with the cell extracts (5 μg of protein) with (lanes 6 to 9) or without (lanes 2 to 5) a 150-fold molar excess of the same unlabeled oligonucleotide, and the complexes formed were analyzed by electrophoresis. No extract was included in lane 1 (F). The arrows indicate the complexes preferentially formed with the 11-, 14-, and 20-h cell extracts.

ing concentrations of KCl to minimize nuclease activity and to concentrate an enhancer-binding factor in a fraction. The fraction which eluted at 0.8 M KCl protected the sequence between −275 and −259, as shown in Fig. 9. The sequence protected corresponds to the sequence which was estimated to contain the enhancer element.

The *cdc2* enhancer activates the basal promoter at the G_1/S boundary. To confirm the ability of the presumed enhancer

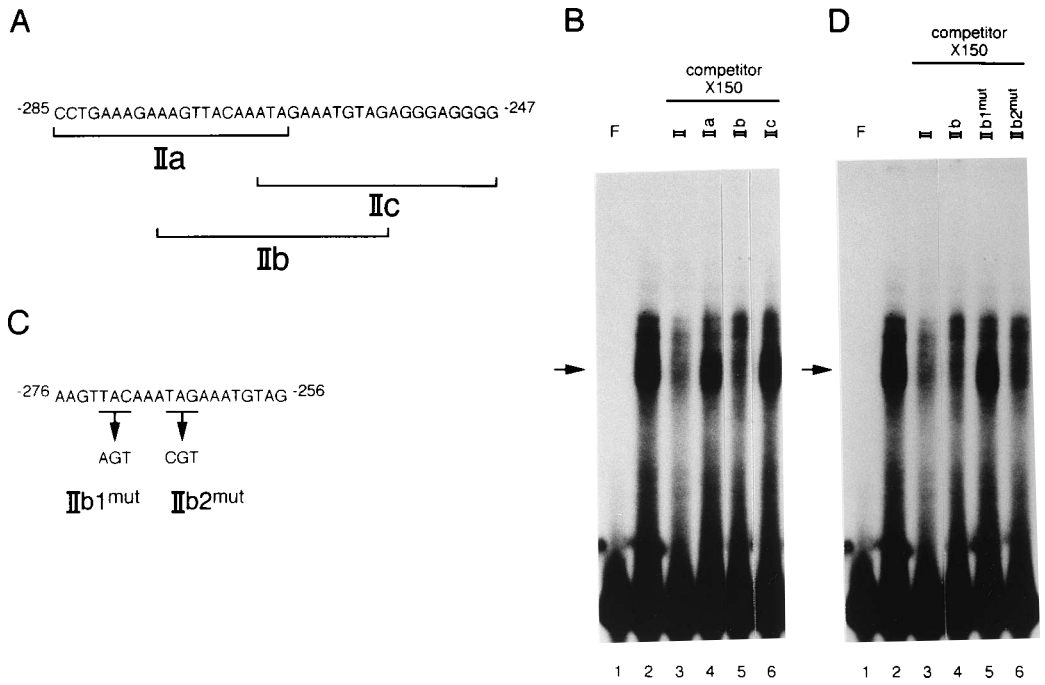

FIG. 7. Effect of base substitutions introduced into enhancer region on complex formation. (A) Oligonucleotides IIa, IIb, and IIc containing the 5′, central, and 3′ portions of oligonucleotide II, respectively, were chemically synthesized and used as competitor oligonucleotides for complex formation. (B) ^{32}P-labeled oligonucleotide II (0.5 fmol) was incubated with extract (5 μg of protein) prepared from quiescent g12-21 cells after serum stimulation for 12 h in the presence and absence of the competitor oligonucleotide, as indicated above each lane. No extract was incubated in lane 1 (F). (C and D) Base substitutions were introduced into oligonucleotide IIb containing the presumed enhancer element as indicated. These oligonucleotides, IIb1^{mut} and IIb2^{mut}, were used as competitors for complex formation as described for panel B.

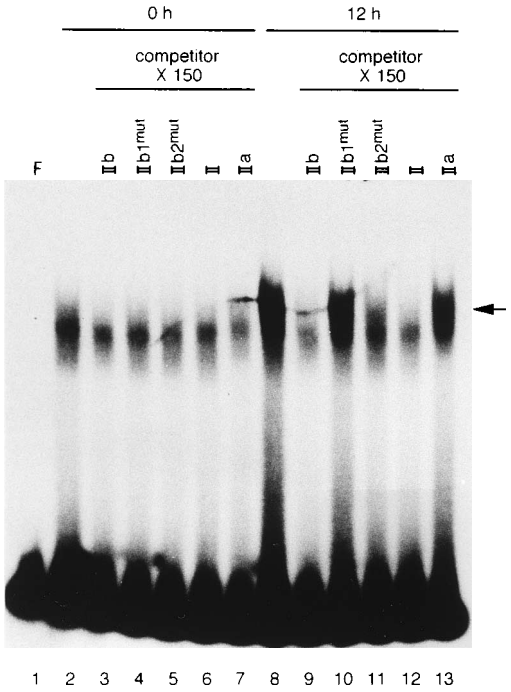

FIG. 8. Complex formation by enhancer element with quiescent cells and growth-stimulated cell extracts. ^{32}P-labeled oligonucleotide IIb (0.5 fmol) was incubated with the extract prepared from quiescent g12-21 cells (0 h) or the cells serum stimulated for 12 h (12 h) in the presence or absence of the competitor oligonucleotides, as indicated above each lane. The arrow indicates the complex preferentially formed with the 12-h extract.

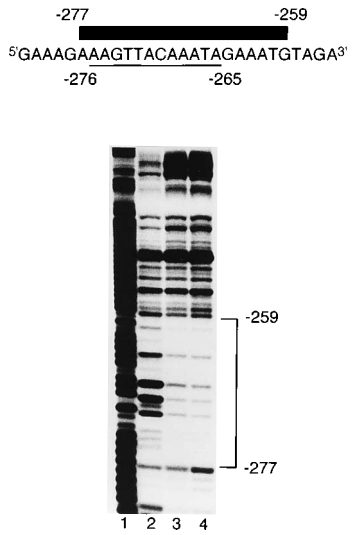

FIG. 9. DNase I footprinting of cdc2 enhancer region formed with growth-stimulated g12-21 cell extract. The oligonucleotide between −314 and −184 was labeled with ^{32}P at the 5′ end of the coding sequence. The oligonucleotide was incubated with the 0.8 M KCl eluate of the serum-stimulated g12-21 cell extract from a heparin-Sepharose CL-6B column and subjected to DNase I footprinting. Lanes: 1, the Maxam-Gilbert A+G chemical cleavage reaction mixture; 2, 80 μg of bovine serum albumin was incubated; 3 and 4, 5- and 10-μg amounts of protein, respectively, in the 0.8 M KCl eluate were incubated. The nucleotide sequence of the protected region and the presumed enhancer element are indicated by a black box and by a thick underline, respectively. The numbers show the nucleotide positions at both ends of the protected region and the enhancer element.

sequence to activate the basal *cdc2* promoter at the G_1/S boundary and to ascertain the role of the E2F motif in enhancer-dependent activation, base substitutions were introduced into the presumed enhancer sequence or the E2F motif or both in pcdc2luc31, as shown in Fig. 10A. These substituted luciferase constructs were transfected to quiescent g12-21 cells and maintained in low-serum medium for 48 h. Luciferase activities were similarly assayed at various times after growth stimulation by serum. All the constructs having the base substitution in the presumed enhancer sequence or the E2F motif or both showed reduced promoter activities at the G_1/S boundary. The sequence $^{-272}$TAC^{-270}, estimated to be essential for an enhancer-binding factor, was also essential for the induction of *cdc2* promoter activity at the G_1/S boundary. The induction is also dependent on the E2F motif.

Since the enhancer element was estimated to reside between −276 and −265, the oligonucleotide containing three repeats of this sequence was synthesized and fused to the 5′ side of the basal promoters of pcdc2luc11 and pcdc2luc19. The former promoter lacks the E2F motif. The induction of luciferase activity by these constructs was analyzed similarly after transfection to quiescent g12-21 cells (Fig. 10B). The linkage of the synthetic enhancer conferred strong inducibility to the basal promoter containing the E2F motif but not to the basal promoter lacking the E2F motif at the G_1/S boundary.

DISCUSSION

The *cdc2* and *CDC28* genes that encode a p34$^{cdc2/CDC28}$ protein kinase were first identified in *Schizosaccharomyces pombe* and *S. cerevisiae* (39, 42) and have essential roles in both the G_1-S and G_2-M transitions of the cell cycle (14). A human homolog of the *cdc2* gene isolated by complementation in yeast cells, however, seems to regulate primarily the G_2-M transition and might not be involved in the G_1-S transition (23, 47, 52). A family of cdc2-related kinases, cdk2, has been shown to play an essential role in the G_1-S transition, since microinjection of an anti-cdk2 antibody in G_1 cells inhibited the entry to S phase, while the anti-cdc2 antibody did not, resulting in cell growth arrest in G_2 phase (47). On the other hand, a role of cdc2 in the G_1-S transition has been proposed for T lymphocytes (16) and Burkitt's lymphoma cells (34). Although cdc2 lacks a clear role in the G_1-S transition, the levels of both the *cdc2* transcript and p34^{cdc2} begin to increase at the G_1/S boundary and are extensively elevated during S phase (25, 43, 53).

To study the mechanism of the induction of cdc2 expression at the G_1-S boundary, the 5′-flanking region of the rat *cdc2* gene was isolated and its structural features were analyzed. The elements required for the induction were analyzed by construction of 5′ sequential deletion derivatives of the promoter fused to a reporter gene and by the formation of complexes in the promoter region containing the presumed enhancer element. Base substitutions were introduced into the presumed enhancer element to ascertain its role in the induction of the promoter activity. These analyses revealed the enhancer element, $^{-276}$AAGTTACAAA^{-267}, which conferred strong inducibility at the G_1/S boundary on the basal *cdc2* promoter. A factor interacting with this sequence was induced after late G_1 phase. This enhancer action depends on the E2F motif located in the basal promoter from positions −130 to −123. Introduction of base substitutions, which convert the sequence TTTCGCGC to TTTCGAAC, severely inhibited promoter activity. The insertion of three repeats of the above enhancer sequence to the 5′ side of the basal promoter containing the E2F motif resulted in a steep increase in the pro-

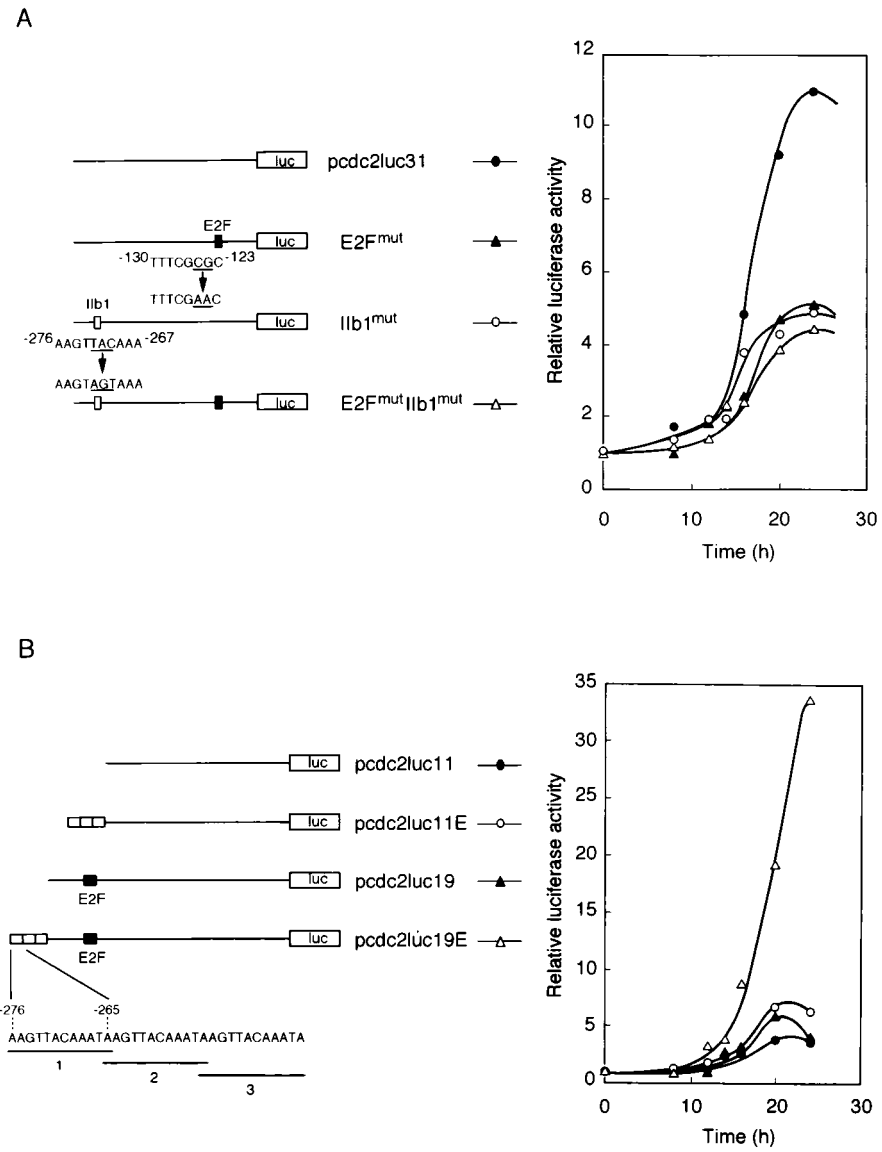

FIG. 10. *cdc2* enhancer stimulates basal promoter activity at G_1/S boundary. (A) Base substitutions were introduced into the enhancer element, the E2F motif, or both in pcdc2luc31, as indicated. Confluent monolayers of 3Y1 cells were transfected with 20 μg each of these derivatives of pcdc2luc31 and maintained in low-serum medium for 48 h. Cell growth was then stimulated by replacing the medium with the fresh medium containing 10% FCS, and luciferase activities were assayed at the times indicated. The activity expressed in the unstimulated cells (0 h) was taken as 1. Average values for three independent experiments are plotted. (B) The oligonucleotide containing three repeats of the enhancer element between −276 and −265 was fused to the 5′ end of the basal promoter in pcdc2luc11 and pcdc2luc19 to generate pcdc2luc11E and pcdc2luc19E, respectively. These luciferase constructs were transfected to quiescent g12-21 cells, and luciferase activities were assayed after growth stimulation by serum as described for panel A.

moter activity at the G_1/S boundary, but insertion into the basal promoter lacking the E2F motif had no effect (Fig. 10B).

The E2F motif in the basal promoter seems to be required not only for the induction of the promoter activity at the G_1/S boundary but also for extensive activation of the promoter activity during S phase, since the induction of the promoter activity by pcdc2luc31 carrying the substituted E2F motif was much delayed and occurred in mid-S phase to a much lesser extent (Fig. 5). We previously showed that the E2F complexes in the *cdc2* promoter were formed predominantly with the extracts prepared from g12-21 cells in late G_1 phase and at the G_1/S boundary but poorly with the extract from the cells in S phase. These results suggest that the function of E2F at the G_1/S boundary may elicit some unknown factor that is required for the extensive activation of the *cdc2* promoter during S phase. The Sp1 motif in the basal promoter is also involved in the induction of the promoter activity at the G_1/S boundary, since the promoter of pcdc2luc31 carrying the substituted Sp1 motif was activated after the cells entered S phase (Fig. 5).

The negative element which resides between −589 and −310 in the upstream sequence of the enhancer element might be required for repression of the *cdc2* promoter during G_1 phase. This negative effect, however, was not completely suppressed at the G_1/S boundary, since the levels of luciferase activities induced by pcdc2luc219 and pcdc2luc58 were consistently lower than that induced by pcdc2luc31, which lacks the negative element (Fig. 4). The induction of *cdc2* promoter activity at the G_1/S boundary may therefore be regulated by the

following factors. (i) A factor induced in late G_1 phase may activate the enhancer activity through interaction with its sequence at the G_1/S boundary and in S phase (Fig. 6–8). (ii) The binding of a factor to the enhancer may alter the conformation of the neighboring region so that the negative action of the upstream sequence is reduced. Alternatively, the binding of a factor to the negative element or the release of a factor from the negative element in late G_1 phase may reduce its negative action. (iii) The E2F motif plays an important role in the enhancer-dependent activation of the promoter at the G_1/S boundary as stated. An active form of free E2F seems to be released during middle to late G_1 phase from the complexes formed with pRB and its related proteins through phosphorylation of these proteins by cyclin D-cdk4 (5, 12, 27, 35) and cyclin E-*cdk2* (7, 29). The activation of the *cdc2* promoter by $E1A_{12S}$ in g12-21 cells after treatment with DEX seems to occur essentially by the same mechanism, since E1A-induced activation of the *cdc2* promoter at the G_1/S boundary was strongly stimulated by the presence of the enhancer and reduced by the presence of the upstream negative element (Fig. 4B). The complex formed at the enhancer sequence with the extracts prepared from DEX-treated g12-21 cells in late G_1 phase, at the G_1/S boundary and in S phase showed the same mobility as that formed with the extracts from the serum-stimulated cells (data not shown). The G_1 phase was shortened because of the acceleration of the G_1-S transition by $E1A_{12S}$, as previously shown (25).

There is about 60% sequence homology in the *cdc2* promoter region up to position −700 between the rat and human genes. The basal promoter sequence between −183 and −122 containing the E box, Sp1, and E2F motifs has especially high degrees of homology with that of the human *cdc2* gene (Fig. 1B). A sequence similar to that of the rat *cdc2* enhancer is also present in the human *cdc2* promoter at a similar position, as shown below:

Rat $^{-276}$AAGTTACAAA^{-267}
Human $^{-278}$AATTTA-AAA^{-270}

Although the human *cdc2* promoter has been analyzed by several groups of investigators, this sequence has not been unraveled, since a deletion derivative of the *cdc2* promoter having the presumed enhancer element but lacking the upstream negative element has not yet been constructed. It has been reported that repression of the human *cdc2* promoter by pRB in cycling cells requires the promoter sequence between −245 and −109 (4). This region retains the E2F motif but lacks the presumed enhancer sequence shown above. The requirement of E2F for the induction of *cdc2* promoter activity was also shown in human hematopoietic cells (17).

The human *cdc2* promoter is also activated by several oncogene products tested thus far. The human *cdc2* promoter up to −465 fused to the CAT gene is activated in hematopoietic cells six- to eightfold by cotransfection with the c-*myb* expression vector (30). The sequence contains the two closely spaced c-*myb* binding sites between −410 and −392. Since this promoter sequence also contains the presumed enhancer sequence, the enhancement might be caused via the enhancer element. Microinjection of expression constructs encoding H-$ras^{V\text{-}12}$ and c-*myc* along with *cdc2* promoter-luciferase reporter plasmids containing the promoter sequence up to position −700 or higher into quiescent REF52 cells led to a threefold increase in *cdc2* promoter activity (2). This enhancement, however, was not observed when a *cdc2* promoter-luciferase construct containing the promoter sequence up to −245 was employed. We suggest that this difference may be caused by the presence or absence of the presumed enhancer element. The human *cdc2* promoter sequence, $^{-278}$AATTTAAAA^{-270} is likely to have enhancer activity, as does the rat *cdc2* enhancer element unraveled in this study.

The results presented here and those obtained by the analyses of the human *cdc2* promoter strongly suggest that the expression of cdc2 is dually regulated at the levels of both transcription and posttranslation. The former is regulated at the G_1/S boundary, and the latter is regulated in G_2 phase.

ACKNOWLEDGMENT

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