Negative Regulation of the Rat cdc2 Promoter in G_1 by the Silencer Element

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Abstract

Expression of the *cdc2* gene is induced steeply at the G_1-S-phase boundary. The previous analysis of promoter elements that confer inducibility revealed the enhancer at positions −276 to −265. Enhancer activity is suppressed by the upstream sequence that seems to contain the silencer. The silencer element was analyzed by fusing several oligonucleotides covering the silencer region upstream of the enhancer in the cdc2 promoter-luciferase construct. Oligonucleotide IV, which suppressed enhancer activity, was further dissected by the introduction of base substitutions and by forming the DNA-protein complexes with quiescent rat cell extract. The silencer element, AAGTAGTAAAAATA, was finally identified at positions −374 to −360, which resembles the enhancer sequencer but contains extra internal AG residues. Silencer complexes were formed with the quiescent cell extract, whereas the amounts of the complexes decreased with the progression of the cell cycle, and nearly no complexes were formed with the late G_1 cell extracts. Conversely, the enhancer complex begins to be formed after late G_1. Among the three silencer complexes, the formation of the slowest-migrating complex (complex III) was inhibited by the enhancer sequence, suggesting that a common factor interacts with both the silencer and enhancer. These results suggest that the conversion of complex formation from the silencer to the enhancer site regulates the induction of cdc2 promoter activity at the G_1-S-phase boundary.

Introduction

The progression of the cell cycle is regulated by stage-specific expression of multiple cyclins and cdks[2] and by phosphorylation of target proteins that drive cell cycle events by these cdks (1). The cdc2/CDC28 gene identified in *Shizosaccharomyces pombe* and *Saccharomyces cerevisiae* (2, 3) is the sole component of cdk in yeasts and is expressed throughout the cell cycle. The function of cdc2/CDC28 is required for both the G_1-S-phase and G_2-M-phase transitions of the cell cycle (4) by associating with different cyclins. In mammalian cells, however, both cyclins and cdks consist of multiple members, and human cdc2 is activated at the end of G_2 by association with cyclin B and by phosphorylation and dephosphorylation of key amino acid residues, triggering the entry into M phase (5, 6). Although cdc2 seems to play no apparent role in the G_1-S-phase transition, its expression is steeply induced at the G_1-S-phase boundary (7–9).

Previous studies on the cdc2 promoter suggested that cdc2 promoter activity is restrained during G_0 and G_1, presumably by pRB and related proteins that sequester transcription factors such as E2F through formation of the complex (10). Phosphorylation of the pRB family members by cyclin D- and cyclin E-dependent kinases releases E2F (11–16), which is essential for maximal induction of the *cdc2* gene at the G_1-S-phase boundary (17).

We recently showed that the activity of rat cdc2 promoter is also regulated by the enhancer element (17). By analyzing the 5′ sequential deletion derivatives of the promoter and their base-substituted variants, the enhancer element, $^{-276}$AAGTTACAAATA^{-265}, which confers inducibility on the basal promoter containing the E2F motif at the G_1-S-phase boundary, was identified. The amount of DNA-protein complex formed at the enhancer sequence with quiescent cell extract was very small, but it increased greatly when extracts were prepared after late G_1. In addition to the enhancer element, there seems to be a silencer sequence upstream of the enhancer, which antagonizes the enhancer function. The silencer is the *cis*-acting elements that exerts a negative effect on promoter activity. Silencer sequences have been found and localized adjacent or distal to the enhancer/promoter sequences in a variety of genes (18). Although the precise mechanism of interaction between the enhancer and silencer has not yet been fully clarified, the binding of a negative factor(s) to the silencer sequence is presumed to prevent the binding of an activating factor(s) to the enhancer sequence by steric hindrance (19–24).

In this study, the silencer sequence presumed to be located upstream of the enhancer was analyzed by synthesizing the oligonucleotides covering the silencer region and by introducing base substitutions into these oligonucleotides. Each oligonucleotide was linked to the 5′ end of the cdc2

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[2] The abbreviations used are: cdK, cyclin-dependent kinase; EMSA, electrophoretic mobility shift assay; pRb, retinoblastoma protein; MCS, multicloning site; PMSF, phenylmethyl sulfonyl fluoride.

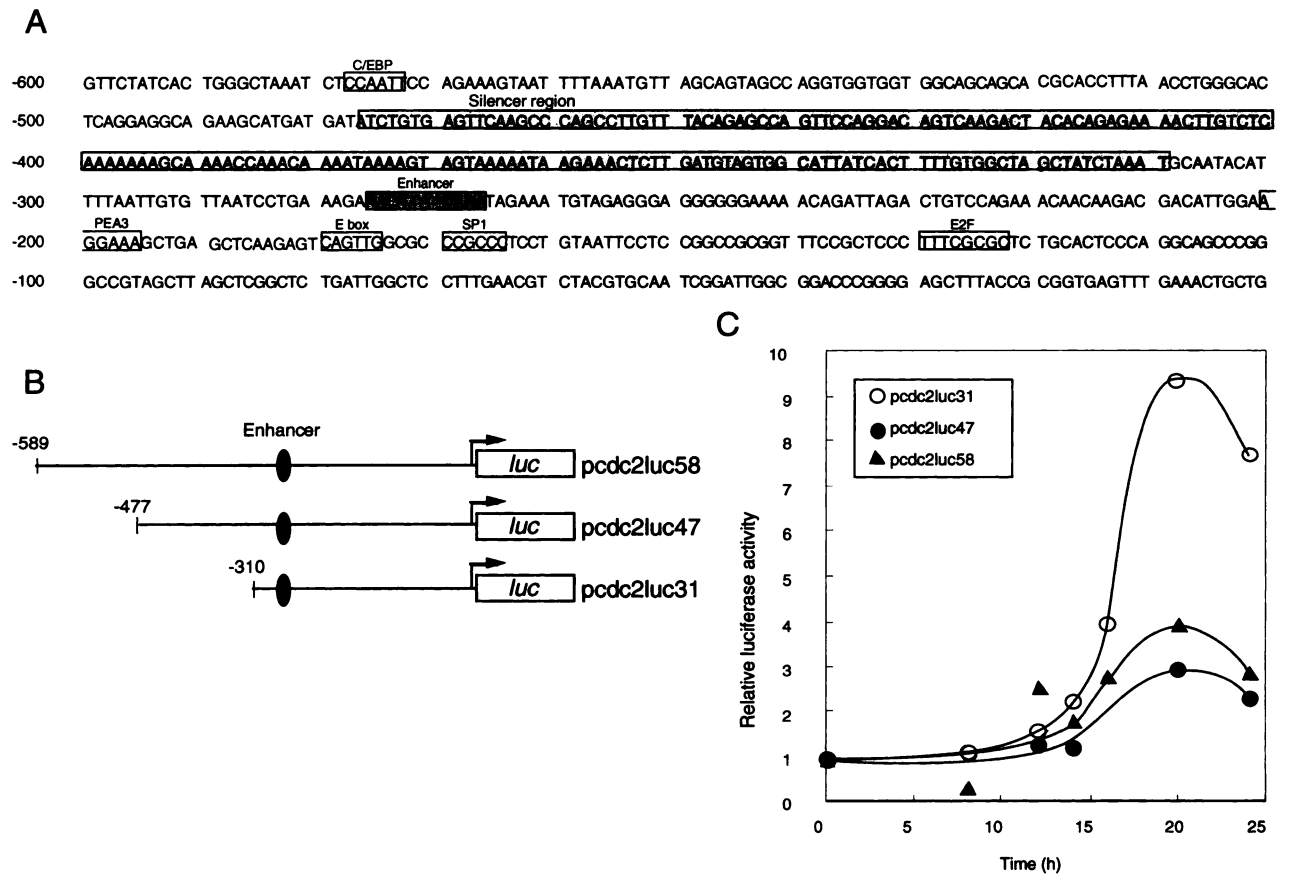
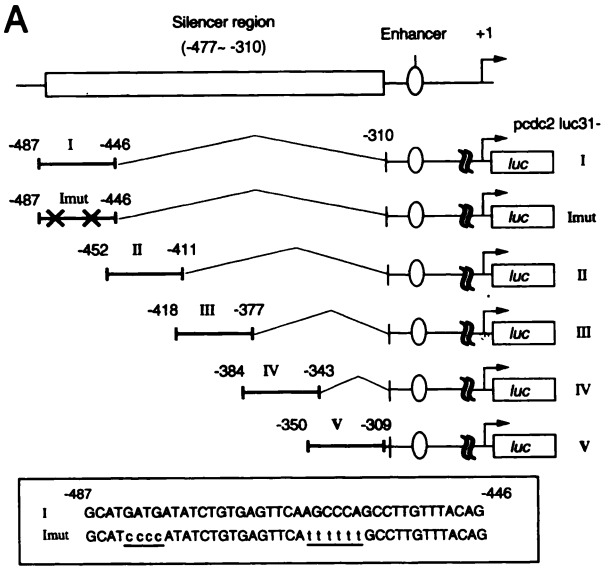


Fig. 1. Suppression of cdc2 promoter activity by the silencer region. *A*, nucleotide sequence of the 5′ flanking region of the rat *cdc2* gene. The positions of consensus sequences for known transcription factors are *boxed*. The silencer region and the enhancer element are shown by the *shaded box*. *B*, structures of the cdc2 promoter-luciferase constructs. The *arrows* indicate the start site of transcription. The *numbers* indicate the 5′ end positions of the promoter sequence. *C*, suppression of cdc2 promoter activation at the G_1-S-phase boundary by the silencer region. Subconfluent monolayers of 3Y1 cells were transfected with 20 μg of the cdc2 promoter-luciferase constructs per 90-mm dish and maintained in low-serum (0.5% FCS) medium for 48 h. Cell growth was then stimulated by replacing the medium with fresh medium containing 10% FCS. Luciferase activities were assayed at the times indicated. The lowest activity expressed by pcdc2luc31 in the unstimulated cells was taken as 1.

enhancer-promoter fused to luciferase cDNA, and its ability to suppress promoter activation at the G_1-S-phase boundary was analyzed. The silencer element was identified at positions −374 to −360, which has a sequence similar to that of the enhancer. The DNA complexes at the silencer site formed abundantly with quiescent rat cell extract but scarcely formed with extracts prepared after late G_1. Conversely, the formation of the complex at the enhancer site with the extracts began to be observed after late G_1 (17). The possible mechanism of silencer action is discussed.

Results

Analysis of the Silencer Region that Suppresses cdc2 Promoter Activity. Our previous analyses of regulatory elements in rat cdc2 promoter with 5′ sequential deletion derivatives of the promoter fused to luciferase cDNA identified the enhancer element AAGTTACAAATA at positions −276 to −265, which confers the inducibility of the promoter at the G_1-S-phase boundary (17). The analyses also suggested the presence of the silencer element upstream between positions −589 and −310, which suppresses the enhancer activity (Fig. 1*A*). To localize the silencer element in this region, three cdc2 promoter-luciferase cDNA constructs, pcdc2luc58, pcdc2luc47, and pcdc2luc31, which contain the upstream sequence up to −589, −477, and −310, respectively, were constructed (Fig. 1*B*). These constructs were transfected to subconfluent monolayers of rat 3Y1 cells and maintained in low-serum (0.5% FCS) medium for 48 h to synchronize the cells in the quiescent (G_0) state. The cells were then growth-stimulated by the addition of fresh medium containing 10% FCS, and luciferase activities were assayed at various times after growth stimulation (Fig. 1*C*). Under these conditions, the rate of [^{3}H]thymidine uptake into the acid-insoluble fraction began to increase after about 13 h of stimulation. The activity of pcdc2luc31 lacking the silencer region was steeply induced at the G_1-S-phase boundary, whereas that of pcdc2luc58 was reduced to less than half. The activity of pcdc2luc47 was reduced even more than that of pcdc2luc58, indicating that the silencer element is localized in the downstream half of the silencer region, as *boxed* in Fig. 1*A*.



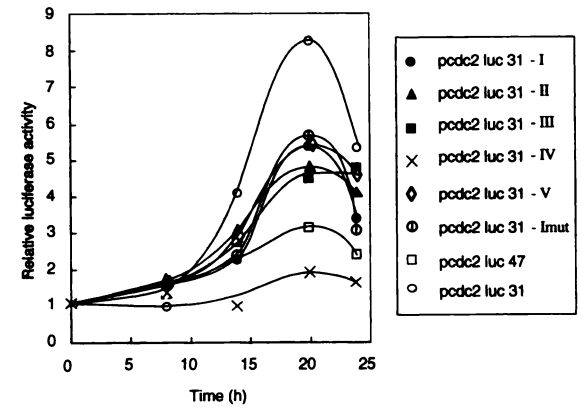


Fig. 2. Dissection of the silencer region with oligonucleotides fused to the cdc2 promoter-luciferase construct. *A*, oligonucleotides I–V covering the silencer region were fused upstream of the enhancer-promoter of pcdc2luc31. The *numbers* indicate the 5′ and 3′ end positions of the oligonucleotides. Oligonucleotide I mut contains 10 base substitutions within the oligonucleotide I, as shown *below* with small letters *underlined*. *B*, the constructs were transfected to subconfluent monolayers of 3Y1 cells, and luciferase activities expressed during G_1-S-phase progression were assayed as described in Fig. 1*B*.

To localize the silencer element further, oligonucleotides I–V covering the silencer region were chemically synthesized and inserted upstream of the enhancer in pcdc2luc31 (Fig. 2*A*). These constructs were similarly transfected to 3Y1 cells, and luciferase activities were assayed after growth stimulation of the quiescent cells by serum (Fig. 2*B*). Among these constructs, the promoter activity of pcdc2luc31-IV was severely suppressed by the oligonucleotide inserted, even more than that of pcdc2luc47, and no significant activation was observed at the G_1-S-phase boundary. The activities of other constructs were reduced to about half, suggesting that sequences I, II, III, and V also exert negative effects on the promoter activity, although their effects are weak. The result suggests that the cdc2 silencer is comprised of multiple modules that independently repress promoter activity, as has been shown in other promoters, *e.g.*, the chicken lysozyme silencer (27). Among these modules, the sequence of oligonucleotide IV has the strongest suppressive activity. As shown below (see Fig. 4), the introduction of base substitutions into oligonucleotide IV reduced the suppressive activity significantly, whereas the introduction of base substitutions into oligonucleotide I had no significant effect (Fig. 2, *A* and *B*).

Effect of Base Substitutions within the Silencer Region on the Formation of DNA-Protein Complexes. A protein(s) that interacts with the silencer element was first analyzed by DNase I footprinting with the 230-bp DNA fragment containing the silencer region and the extract prepared from quiescent 3Y1 cells. Although the footprint obtained was ambiguous, the ladder of DNA fragments generated by DNase I digestion was significantly altered at positions −369 and −361 as compared with that of the control, in which BSA was used instead of cell extract (data not shown). Base substitutions were therefore introduced into oligonucleotide IV at positions −371 to −369 (oligonucleotide IV mut1) and at positions −363 to −361 (oligonucleotide IV mut2) as shown in Fig. 3*A*. The DNA-protein complexes formed within this region were analyzed by EMSAs with the ^{32}P-labeled wild-type and base-substituted oligonucleotide IV and quiescent cell extract (Fig. 3*B*). Oligonucleotide IV formed three complexes designated I, II, and III (Fig. 3*B*, *Lane 2*). Oligonucleotide IV mut2 also formed three complexes (Fig. 3*B*, *Lane 4*), although complex III moved slightly faster than that formed with oligonucleotide IV. In contrast, oligonucleotide IV mut1 formed only complex III, suggesting that complexes I and II are formed at the $^{-371}$TAG^{-369} site, and that complex III is formed at the other site. The specificity of complex formation was also analyzed by competition with unlabeled oligonucleotide IV, oligonucleotide IV mut1, and oligonucleotide IV mut2 (Fig. 3*C*). The formation of complexes with ^{32}P-labeled oligonucleotide IV was almost completely abolished by the presence of an excess of the same unlabeled oligonucleotide IV, but not by oligonucleotide IV mut1 (Fig. 3*C*, *Lanes 3* and *4*). The presence of oligonucleotide IV mut2 also abolished the formation of complexes I and II, but not complex III (Fig. 3*C*, *Lane 5*). The result also indicates that the $^{-371}$TAG^{-369} site is involved in the formation of complexes I and II.

To see which sides of the sequence around $^{-371}$TAG^{-369} are required for the formation of the complexes and to analyze the involvement of the sequence $^{-363}$ATA^{-361} further, three 15-bp oligonucleotides, IV-A, IV-B, and IV-C, which contain different lengths of nucleotide sequences in either side of $^{-371}$TAG^{-369}, were synthesized as shown in Fig. 3*A*. Only oligonucleotide IV-A contains the sequence $^{-363}$ATA^{-361}. These oligonucleotides were used as competitors for the formation of complexes with ^{32}P-labeled oligonucleotide IV. As shown in Fig. 3*D*, oligonucleotide IV-A, but not oligonucleotides IV-B and IV-C, inhibited the formation of complexes I and II to an extent similar to that caused by unlabeled oligonucleotide IV, suggesting that the TA stretch located 3′ of $^{-371}$TAG^{-369} is important for complex forma-

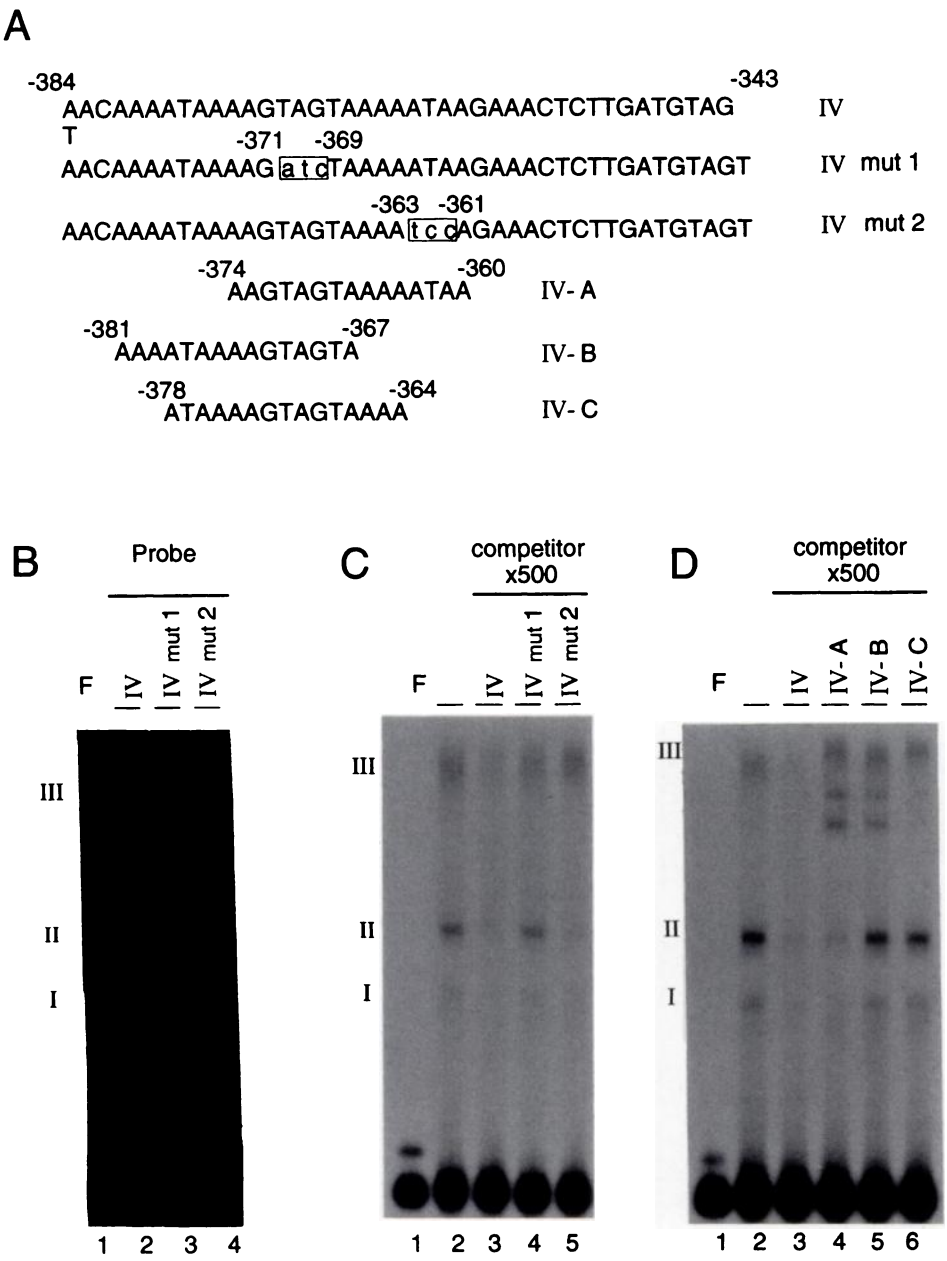


Fig. 3. Formation of DNA-protein complexes with the oligonucleotide carrying the presumed silencer sequence. *A*, nucleotide sequences of the oligonucleotides used for EMSAs. The oligonucleotides IV mut1 and IV mut2 carry base substitutions in the presumed silencer sequence as indicated. Oligonucleotides IV-A, IV-B, and IV-C were used as competitors for complex formation with oligonucleotide IV. *B*, the complexes were formed with ^{32}P-labeled oligonucleotides IV, IV mut1, and IV mut2, as indicated *above* each lane, and the extract prepared from quiescent 3Y1 cells. The complexes formed were analyzed by EMSA. No extract was included in *Lane 1* (*F*). *C*, the complexes were formed with ^{32}P-labeled oligonucleotide IV and the quiescent cell extract in the presence of a 500-fold molar excess of unlabeled oligonucleotides as indicated *above* each lane. *D*, the complexes were formed as described in *C* in the presence of a 500-fold molar excess of unlabeled oligonucleotides IV-A, IV-B, and IV-C, as indicated *above* each lane.

tion, although the base substitution within the TA stretch ($^{-363}$ATA^{-361} → $^{-363}$TCC^{-361}) had little effect. In the presence of unlabeled oligonucleotides IV-A and IV-B, two extra complexes that migrated slightly faster than complex III were formed, and migration of complex III was delayed. These extra bands may be generated by the interaction of ^{32}P-labeled oligonucleotide IV with the 15-bp small oligonucleotides IV-A and IV-B, although its precise mechanism is presently unclear.

Identification of the Silencer Element by Introduction of Base Substitutions into pcdc2luc47. To correlate the sequence required for complex formation with that required for suppression of promoter activity at the G_1-S-phase boundary, the same base substitutions were introduced into the promoter of pcdc2luc47 at $^{-371}$TAG^{-369} or $^{-363}$ATA^{-361} to generate pcdc2luc47m1 and pcdc2luc47m2 as shown in Fig. 4*A*. These constructs were transfected to subconfluent monolayers of 3Y1 cells simultaneously with the control constructs, pcdc2luc47 and pcdc2luc31, and luciferase activities were similarly assayed after growth stimulation of the quiescent cells (Fig. 4*B*). The promoter activity of pcdc2luc31 was steeply induced at the G_1-S-phase boundary, whereas the promoter activation of pcdc2luc47 containing the silencer region was reduced to less than half, as observed in Fig. 1*B*. Under these conditions, the promoter activity of pcdc2luc47m1 was reduced slightly as compared with that of pcdc2luc31, whereas the activity of pcdc2luc47m2 was reduced considerably but was still higher than that of

pcdc2luc47. The result indicates that the sequence $^{-371}$TAG^{-369} is primarily involved in suppression of the cdc2 promoter activation at the G_1-S-phase boundary, but the sequence $^{-363}$ATA^{-361} is also involved in suppression, although its effect is weaker than that of $^{-371}$TAG^{-369}. The sequence required for the suppression of promoter activity is therefore correlated with that required for the formation of complexes I and II. Based on these results, we defined the sequence of oligonucleotide IV-A as the silencer element.

We previously showed that activation of the cdc2 promoter at the G_1-S-phase boundary is strictly dependent on the E2F site present at positions −130 to −123, and the introduction of base substitutions into the E2F motif resulted not only in a severe reduction in promoter activity but also in a significant delay in activation to mid-S phase (17). The presence of the silencer element upstream of the enhancer reduced the extent of promoter activation without shifting the peak to mid-S phase. The result suggests that the silencer suppresses the E2F site-dependent enhancer activity.

The DNA-Protein Complexes Formed at the Silencer Sequence Diminish with G_1 Progression. The previous study on the formation of DNA-protein complex at the enhancer sequence by EMSAs showed that the complex scarcely formed with quiescent 3Y1 cell extract but formed abundantly with cell extracts prepared after late G_1 (17). To compare the changes in complex formation at the silencer and enhancer sites, complex formation was analyzed with oligonucleotide IV (positions −384 to −343) and E oligonucleotide (positions −280 to 259) containing the enhancer element. The extracts were prepared from quiescent 3Y1 cells after serum stimulation for 0, 6, 11, 14, and 20 h (Fig. 5*A*). These stages correspond to G_0, mid-G_1, late-G_1, the G_1-S-phase boundary, and S phase, respectively. Four complexes were formed with oligonucleotide IV and the 0-h extract. The slow-migrating complexes designated IIIa and IIIb seemed to be the same as the broad complex III observed in Fig. 3, in which the complexes IIIa and IIIb migrated more closely. Complex II was predominant. The amounts of complexes I and II formed with the 6-h extract were reduced slightly but were greatly reduced with the 11-h extract. Nearly no complexes I and II were formed with the 14- and 20-h extracts, indicating that a protein factor(s) that forms the complexes with the silencer site began to decrease in mid-G_1 and was scarcely present after late G_1. The amounts of complexes IIIa and IIIb formed with cell extracts were unchanged until late G_1 but decreased steeply after the G_1-S-phase boundary. The reduction in the amounts of complex II during G_1-S-phase progression was quantitated with a densitometer and plotted in Fig. 5*B*. In contrast, the complex was not formed significantly with E oligonucleotide containing the enhancer and extracts prepared before mid-G_1 but formed with extracts prepared at late G_1 to the G_1-S-phase boundary. As previously shown, complex formation was abolished by the presence of an excess of the same unlabeled oligonucleotide, but not by the oligonucleotide containing the base-substituted enhancer (17). The result indicates that complex formation at the silencer and enhancer sites in the cdc2 promoter is mutually exclusive. The formation of the complex at the enhancer site seems to begin

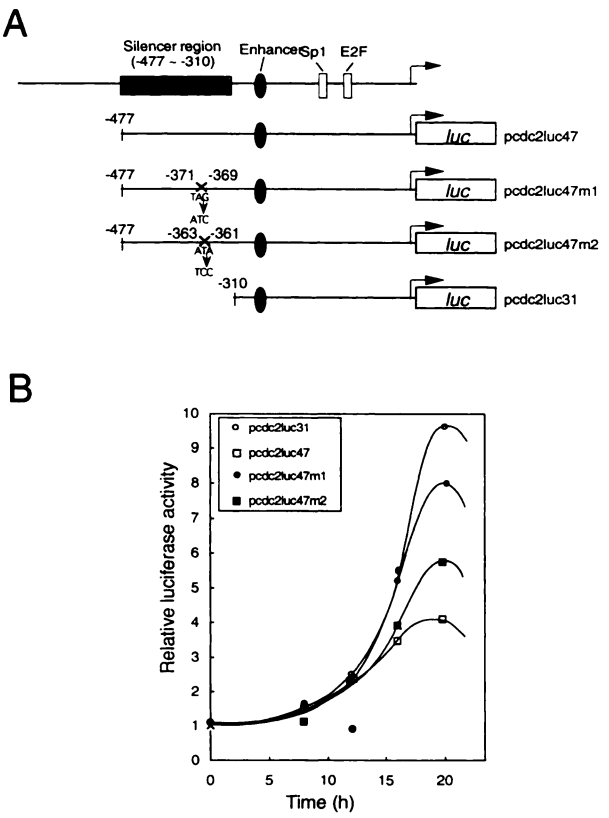


Fig. 4. Effect of the base substitution in the presumed silencer sequence on cdc2 promoter activity. *A*, structures of pcdc2luc47 mut1 and pcdc2luc47 mut2 carrying the base substitution in the presumed silencer sequence. *B*, subconfluent monolayers of 3Y1 cells were transfected with the pcdc2luc constructs shown in *A*, and luciferase activities were assayed as described in Fig. 1*B*.

with the dissociation of complexes formed at the silencer site after late G_1, permitting activation of the cdc2 promoter at the G_1-S-phase boundary.

Competitive Formation of the Complexes at the Silencer and Enhancer Sites. The similarity of the sequences between the silencer and enhancer elements (Fig. 6) suggested that a particular factor may bind to both of these sequences and may bind its modified form to either of them. To analyze this possibility, the complexes were formed with the ^{32}P-labeled 27-bp oligonucleotide containing either the enhancer element (E oligonucleotide) or the silencer element (S oligonucleotide) and 3Y1 cell extracts in the presence of these unlabeled oligonucleotides (Fig. 7). The formation of the complex with E oligonucleotide and the 11-h extract prepared from late G_1 cells was inhibited significantly by the presence of a 10-fold molar excess of unlabeled E oligonucleotide and further inhibited by the presence of a 100-fold molar excess of the oligonucleotide (Fig. 7*A*, *Lanes 9–11*). Under these conditions, the presence of the unlabeled S oligonucleotide and the Smut oligonucleotide containing six base substitutions within the silencer element (Fig. 7*C*) had no effect (Fig. 7*A*, *Lanes 3–8*). The formation of complexes I, II, and III with S oligonucleotide and the 0-h extract prepared from quiescent cells was completely abolished by the pres-

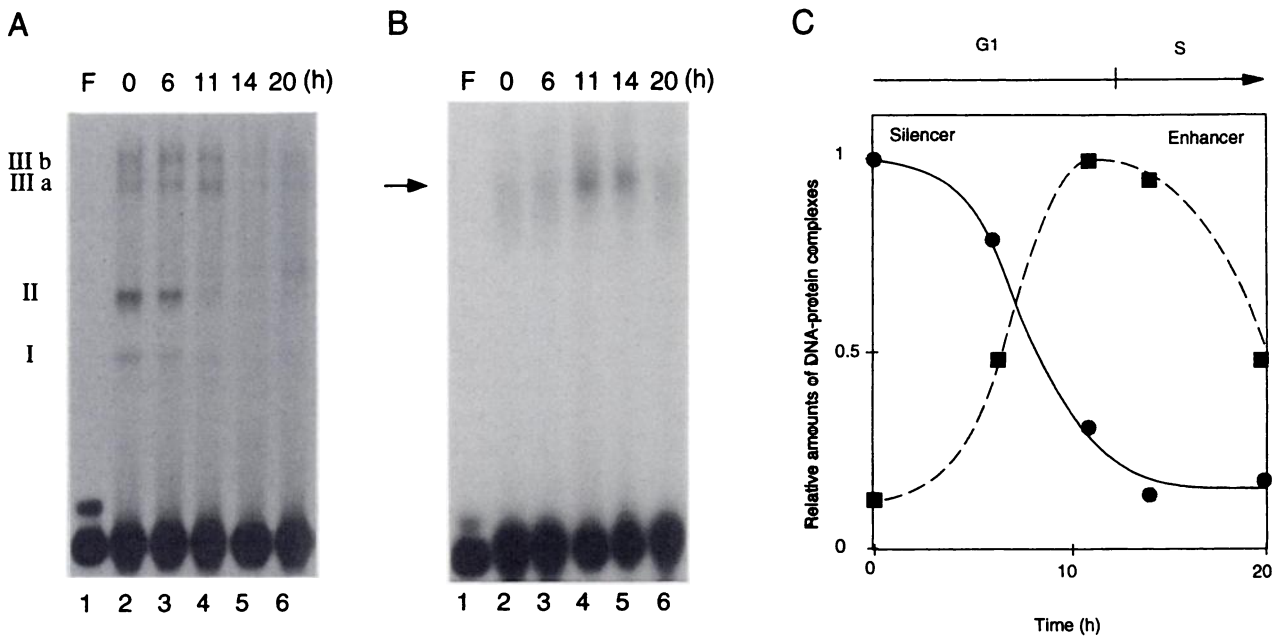


Fig. 5. Formation of DNA-protein complexes at the silencer site diminishes after late G_1. ^{32}P-labeled oligonucleotide IV containing the silencer element (*A*) and ^{32}P-labeled E oligonucleotide containing the enhancer element were incubated with cell extracts prepared from quiescent 3Y1 cells (*0 h*) and from cells that were growth-stimulated by serum for 6 (mid-G_1), 11 (late G_1), 14 (G_1-S-phase boundary) and 20 h (S phase), as indicated *above* each lane. The complexes formed were analyzed by EMSAs. No extract was included in *Lane 1* (*F*). *C*, the amounts of complex II formed with oligonucleotide IV and the complex formed with E oligonucleotide were quantitated with a densitometer, and their relative amounts were plotted.

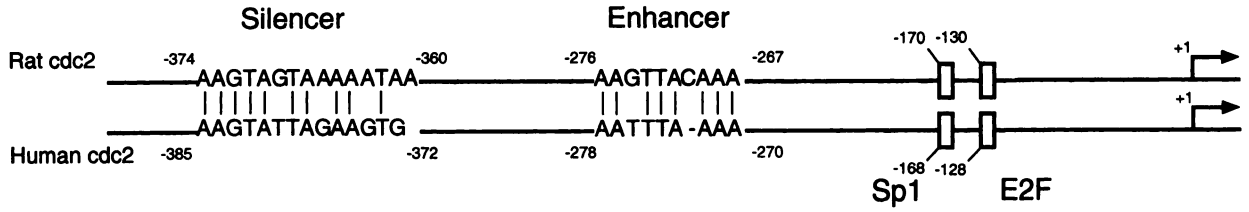
ence of a 100-fold molar excess of the unlabeled S oligonucleotide, but not by the unlabeled Smut oligonucleotide (Fig. 7*B*, *Lanes 3–8*). Interestingly, the formation of complex III, but not complexes I and II, was completely abolished by the presence of a 100-fold molar excess of unlabeled E oligonucleotide (Fig. 7*B*, *Lane 11*). In addition, complex III showed mobility similar to that of the complex formed with E oligonucleotide. Inhibition of complex III formation at the silencer site by the enhancer sequence is consistent with the result obtained in Fig. 3*B*, which showed that complexes I and II are formed at the $^{-371}$TAG^{-369} site, but complex III is formed at the other site. The enhancer contains a sequence similar to that of the silencer but lacks two bases at the TAG site (Fig. 6*B*). These results suggest that at least two factors associate with the silencer: (*a*) one, which is specific to the silencer, binds to the $^{-371}$TAG^{-369} site and forms complexes I and II; and (*b*) the other binds to the other sequence, which is similar to that of the enhancer, and forms complex III. The times of dissociation of the silencer complexes during G_1 progression were also different between complexes I and II and complex III, as shown in Fig. 5*A*.

Discussion

The *cdc2* gene is involved in the G_1-S-phase and G_2-M-phase transitions of the cell cycle in yeasts (4) but seems to be involved only in the G_2-M-phase transition in mammalian cells. cdc2 is maximally activated at the end of G_2 by association with cyclin B and by phosphorylation and dephosphorylation of key amino acid residues of cdc2, triggering entry into M phase (5, 6). Nevertheless, the expression of cdc2 is induced at the G_1-S-phase boundary (7–9). This activation is partly caused by the release of transcription factor E2F from the inactive complex formed with the pRB family members (10) through phosphorylation of these proteins by cyclin D- and cyclin E-dependent kinases (11–16). Our previous studies showed that the expression of the rat *cdc2* gene is also regulated by the enhancer located at positions −276 to −265 in an E2F-dependent manner and is essential for activation of the promoter at the G_1-S-phase boundary (17). In addition, the silencer element that antagonizes enhancer activity seems to be present upstream of the enhancer.

In the present study, the silencer element was identified by linking the synthetic oligonucleotides covering the silencer region upstream of the cdc2 enhancer-proximal promoter fused to the luciferase cDNA and by testing their abilities to suppress enhancer activity at the G_1-S-phase boundary in transfected cells. Oligonucleotide IV from positions −384 to −343, which suppressed enhancer activity completely, was further dissected by the introduction of three base substitutions at two sites, and the silencer element was finally localized at positions −374 to −360. The sequence similar to this silencer is also present in the corresponding position of the human cdc2 promoter, as shown in Fig. 6*A*. Although the third base of the critical TAG sequence in the rat silencer is altered to TAT in the presumed human silencer, 9 of 11 bases from positions −374 to −364 in the rat silencer are identical in the human sequence. The human cdc2 promoter also contains the presumed enhancer sequence at positions −278 to −270, similar to the position in the rat cdc2 pro-

A



B

rat silencer AAGTAGTAAAAATAA

rat enhancer AAGT--TACAAA

Fig. 6. Comparison of rat cdc2 silencer and enhancer sequences with the corresponding sequences in human cdc2 promoter. *A*, the *numbers* indicate the positions of the rat silencer and enhancer sequences and the presumed human silencer and enhancer sequences. The identical bases between the rat and human promoter sequences are shown by the *bars*. *B*, comparison of the base sequences between the rat silencer and enhancer.

moter, and 8 of 10 bases in the rat enhancer sequence are identical in the human sequence (17). These results suggest that the silencer and enhancer elements might be conserved in mammalian cells and work together to regulate the G_1-S-phase transition.

The formation of the DNA-protein complexes at the silencer and enhancer sites seem to be mutually exclusive, and the extract prepared from quiescent (G_0) 3Y1 cells formed the complexes abundantly, with the silencer sequence but not with the enhancer sequence, whereas the cell extracts prepared after late G_1 formed the complex predominantly with the enhancer sequence, but not with the silencer. The result suggests that the enhancer complex began to be formed in late G_1, with the dissociation of the silencer complex that began after mid-G_1. The silencer sequence resembles the enhancer sequence, and 8 of 10 bps are identical between these sequences (Fig. 6*B*). The absence of 2 bps within the critical $^{-371}$TAG^{-369} sequence in the enhancer, however, suggests that the absence is correlated with their difference in function. As shown in Fig. 3*B*, the silencer complexes I and II were formed at the TAG site, whereas complex III was formed at the other site, presumably within the silencer sequence, where the 10-base sequence is identical (except for 1 base) with that of the enhancer. Reflecting this similarity, only the formation of complex III was inhibited by the presence of the enhancer sequence (Fig. 7*B*). The results suggest that at least two factors bind to the silencer sequence, one of which also binds to the enhancer. Modification of the factor may alter the binding preference to these similar sites. We have recently cloned two types of cDNAs encoding the enhancer-binding proteins with related amino acid sequences. Western blot analysis of these proteins for expression during G_1-S-phase progression indicated that they consist of multiple species, and some of them disappear after late G_1, suggesting that posttranscriptional modification, *e.g.*, phosphorylation or dephosphorylation of these protein factors, may be crucial for their abilities to bind DNA.

In addition to negative regulation of cell cycle progression by the pRB family members, the silencer may also function to prevent cells from premature expression of cdc2. Reorganization of the complexes from the silencer to the enhancer sites may result in a conformational change in the cdc2 promoter region, ensuring the induction of the enhancer function. Although the DNA-protein complex was not formed at the silencer site with the cell extracts prepared after late G_1, the presence of the silencer upstream of the enhancer significantly reduced the extent of promoter activation at the G_1-S-phase boundary (Fig. 1), suggesting that the interaction of enhancer-binding proteins may be affected by the conformational change elicited by the silencer sequence. The silencer sequence identified at positions −374 to −360 exerted the strongest negative effect on cdc2 promoter activity; however, the neighboring sequences contained in oligonucleotides I, II, III, and V also showed negative effects on the promoter, although their effects were weak (Fig. 2). The result suggests that the fusion of unsuitable sequences to the basal promoter more or less perturbs the regular conformation of the promoter that is elicited by the binding of various transcription factors, or the silencer region is comprised of multiple modules that independently repress promoter activity, as previously shown in other promoters (25–28). In spite of the negative effect of the silencer on cdc2 promoter activation at the G_1-S-phase boundary, the silencer may be required for cells at the sacrifice of a safety guard for negative regulation of cell cycle progression.

Materials and Methods

Cell Lines. The 3Y1-B cell line, clone 1-6, is a clonal line of Fischer rat embryo fibroblasts (29). The cells were cultivated at 37°C in DMEM with 10% FCS.

Construction of cdc2 Promoter-Luciferase Fusion Plasmids. Construction of pcdc2luc58 and pcdc2luc31, in which the rat cdc2 promoter sequences from positions −589 to 64 and −310 to +64 were fused to the luciferase cDNA, was described previously (17). To construct pcdc2luc47, pcdc2luc58 was cleaved with *Eco*RV at position −477, ligated to the *Bgl*II linker, and circularized after cleavage with *Bgl*II. The DNA was then cleaved with *Bgl*II and *Xho*I, and the DNA fragment from positions −477 to 64 was inserted at the *Kpn*I-*Nhe*I site in the MCS of PGV-B that resides upstream of luciferase cDNA by using the *Kpn*I-*Bgl*II adapter (5′-GATCG-TAC-3′). The cohesive ends of *Xba*I and *Nhe*I share the same sequence

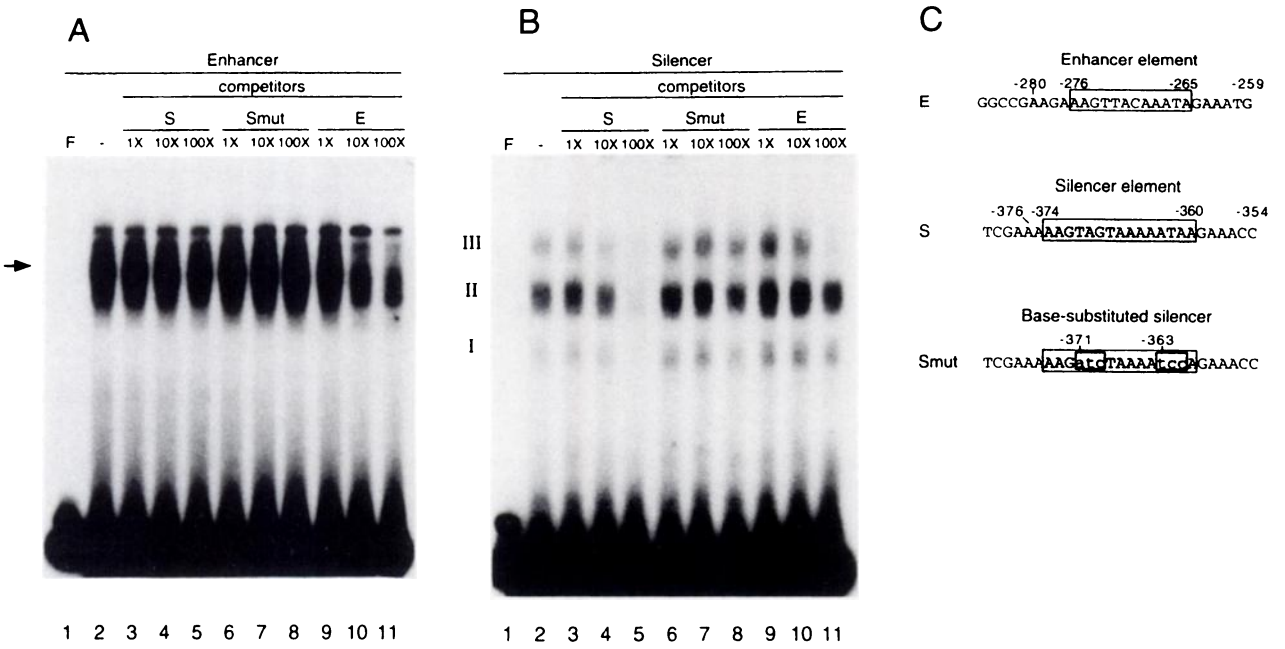


Fig. 7. Competitive formation of the complexes at the silencer and enhancer sites. *A*, the ^{32}P-labeled 27-bp oligonucleotide containing the enhancer element (E oligonucleotide; 1 fmol) was incubated with 5 μg of protein in 11-h extract prepared from late G_1 3Y1 cells in the presence of 1- to 100-fold molar excesses of the unlabeled S oligonucleotide containing the silencer element, Smut oligonucleotide containing the base-substituted silencer, or the E oligonucleotide, as indicated *above* each *lane*. *B*, the ^{32}P-labeled 27-bp S oligonucleotide (1 fmol) was incubated with 5 μg of protein in the 0-h extract from the quiescent cells in the presence of 1- to 100-fold molar excesses of the unlabeled S, Smut, or E oligonucleotide, as indicated *above* each lane. The complexes formed were analyzed by EMSAs. No extract was included in *Lane 1* (*F*), and no competitive oligonucleotide was included in *Lane 2*. *C*, the sequences of the E, S, and Smut oligonucleotide are shown. The enhancer and silencer elements are *boxed*.

(5′-CTAG-3′). To insert oligonucleotides I–V covering the silencer region (Fig. 2*A*) upstream of the cdc2 enhancer-promoter, plasmid PGV-B was cleaved with *Xho*I and *Hin*dIII in MCS and circularized after both ends were blunted to disrupt these restriction sites. The circularized DNA was cleaved with *Sma*I in MCS, and the *Xho*I linker was inserted to create the new *Xho*I site upstream of the cloning site. The DNA was then cleaved with *Kpn*I and *Nhe*I in MCS, and the *Bgl*II-*Nhe*I fragment containing the cdc2 promoter sequence from −310 to 64 was inserted by using the *Bgl*II-*Kpn*I adapter (5′-GATCGTAC-3′) to generate pcdc2luc31. The DNA was then cleaved with *Xho*I upstream of the inserted promoter, and each of the oligonucleotides I–V containing the *Xho*I cohesive ends at both sides was inserted to generate pcdc2luc31-I to -V (Fig. 2*A*). The oligonucleotide I mut containing 10 base substitutions, as shown in the bottom of Fig. 2*A*, was similarly inserted at the *Xho*I site of pcdc2luc31.

Construction of cdc2 Mutant Promoters with Base Substitutions in the Silencer Region. To introduce base substitutions in the silencer region of pcdc2luc47, the 5′ flanking sequence between positions −686 and −343 was amplified by PCR (30) by using pcdc2CAT141 DNA as the template, the upstream sense strand primer from positions −686 to −666, and the downstream antisense strand primer from positions −384 to −343. The downstream primer contains the presumed silencer sequence in which the base substitution ^{371}TAG ^{369} to ATC or ^{363}ATA ^{361} to TCC (Fig. 3*A*) was introduced (31, 32). The product was used as the upstream sense strand primer (a megaprimer; Ref. 32), and asymmetrical PCR was performed using only the megaprimer for five cycles. The downstream antisense strand primer from positions −268 to −248 was then added, and the DNA fragment between positions −686 and −248 was amplified by the second PCR. The product was cleaved with *Eco*RV and *Nhe*I at positions −477 and −323, respectively, and the *Eco*RV-*Nhe*I fragment was inserted at the *Sma*I-*Nhe*I site of pcdc2luc47 to generate pcdc2luc47mut1 and pcdc2luc47mut2 (Fig. 4*A*).

Transient Transfection and Analysis of Gene Expression. DNA transfection was performed by the $CaPO_4$ coprecipitation procedure (33) as modified by Chen and Okayama (34). For analysis of cdc2 promoter activity during G_1-S-phase progression, subconfluent monolayers 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 48–54 h. Cell growth was then stimulated by replacing the medium with fresh medium containing 10% FCS. The cells harvested at various intervals were assayed for luciferase activity with 200 μg of protein from the cell extract and 100 μl of luciferin substrate (Nippongene) with a LB9501 luminometer (Berthold; Ref. 35).

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

Preparation of Whole-Cell Extract. Whole-cell extracts were prepared essentially according to the method of Manley *et al.* (36). 3Y1 cells were washed in PBS containing 0.5 mM $MgCl_2$ and suspended in 4 volumes of hypotonic buffer [10 mM Tris-HCl (pH 7.9 at 4°C), 1 mM EDTA, 5 mM DTT, and 0.5 mM PMSF]. After 20 min, the cells were homogenized, and 4 volumes of sucrose-glycerol solution [50 mM Tris-HCl (pH 7.9 at 4°C), 10 mM $MgCl_2$, 25% (w/v) sucrose, 50% (v/v) glycerol, 2 mM DTT, and 0.5 mM PMSF] were 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 $(NH_4)_2SO_4$ was added to the supernatant to a final concentration of 0.33 g/ml, and the suspension was centrifuged (19,200 rpm) in a Hitachi RP65T rotor for 30 min. The precipitate was dissolved in a minimal volume of buffer D [20 mM HEPES (pH 7.9), 12.5 mM $MgCl_2$, 0.1 mM EDTA, 20% (v/v) glycerol, 2 mM DTT, and 0.5 mM PMSF) containing 0.1 M KCl. The sample was dialyzed against two changes of 1 liter each of buffer D 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.

EMSAs. DNA-protein complexes were formed in a 10-μl reaction mixture (37) containing 20 mM HEPES buffer (pH 7.9), 100 mM KCl, 12.5 mM $MgCl_2$, 1 mM EDTA, 20% (v/v) glycerol, 2 mM DTT, 0.5 mM PMSF, 1 μg of poly(deoxyinosinic-deoxycytidylic acid) · poly(deoxyinosinic-deoxycytidylic acid, 0.5–1.0 fmol (approximately 5×10^3 cpm) of ^{32}P-labeled oligonucleotide, and the whole-cell extract (5 μg of protein) 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-HCl (pH 8.0), 192 mM glycine, and 2 mM EDTA]. The gels were dried and autoradiographed with an intensifying screen at −80°C.

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