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A Role for a Bent DNA Structure in E2F-Mediated Transcription Activation

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We examined the role of promoter architecture, as well as that of the DNA-bending capacity of the E2F transcription factor family, in the activation of transcription. DNA phasing analysis revealed that a consensus E2F site in the E2F1 promoter possesses an inherent bend with a net magnitude of 40 ± 2° and with an orientation toward the major groove relative to the center of the E2F site. The inherent DNA bend is reversed upon binding of E2F, generating a net bend with a magnitude of 25 ± 3° oriented toward the minor groove relative to the center of the E2F site. We also found that three members of the E2F family, in conjunction with the DP1 protein, bend the DNA toward the minor groove, suggesting that DNA bending is a characteristic of the entire E2F family. The Rb-E2F complex, on the other hand, does not reverse the intrinsic DNA bend. Analysis of a series of E2F1 deletion mutants defined E2F1 sequences which are not required for DNA binding but are necessary for the DNA-bending capacity of E2F. An internal region of E2F1, previously termed the marked box, which is highly homologous among E2F family members, was particularly important in DNA bending. We also found that a bent DNA structure can be a contributory component in the activation of the E2F1 promoter but is not critical in the repression of that promoter in quiescent cells. This finding suggests that E2F exhibits characteristics typical of modular transcription factors, with independent DNA-binding and transcriptional activation functions, but also has features of architectural factors that alter DNA structure.

The study of transcriptional regulatory mechanisms has demonstrated the critical role for promoter-binding proteins that are essential for full activity of a given gene. Moreover, various studies have detailed the modular nature of these transcription factors, showing many of them to be composed of independent domains which define promoter specificity and activate transcription (28, 39). This modular view of transcription factors is supported by numerous domain switching experiments whereby promoter specificity domains generally function by specific DNA binding or through protein-to-protein interactions with DNA-bound proteins (28, 39) and transactivation domains are thought to function through protein-protein interactions (24, 38, 47).

More recent work has revealed an apparently distinct class of transcription factors which might be termed architectural components (36). These proteins bind promoter DNA and influence promoter activity by altering the DNA topology, apparently to allow other DNA-bound proteins to functionally interact. One well-characterized example is transcription factor YY1, which upon binding to its DNA recognition sequences, significantly bends the promoter DNA. Depending on the context of sites in the promoter, this perturbation has either a positive or a negative affect on transcription (40). It has been postulated that the role of YY-1 with respect to c-*fos* promoter activity is to bend DNA and allow the synergistic interactions between promoter-bound transcription factors which, in the absence of YY-1, would not be able to interact. Other mammalian transcription factors have been found to bend DNA, including Fos and Jun (15, 30), NF-κB (44), Myc-Max (9), and Oct-1 (51). Although the functional relevance of the DNA bend has not been documented in these cases, the general observation that many transcription factors bend DNA suggests that DNA bending could be particularly important in synergistic regulation of many promoters.

An apparently distinct example of architectural transcription factors are the high-mobility-group (HMG) proteins, which include the lymphoid cell-specific RNA polymerase II factor LEF-1 (49). LEF-1 binds the minor groove of DNA (11, 50) and induces a sharp bend in the DNA double helix (12). In the absence of other upstream elements, multimerized LEF-1 sites have no effect on the transcriptional activity of a basal promoter. However, in the context of the T-cell receptor α enhancer, it appears that the DNA bend induced by LEF-1 allows a functional interaction between transcription factors bound on either side of the LEF-1 site (49, 52). Thus, LEF-1 appears to enhance transcription by facilitating the interaction of other promoter-bound factors. A similar example can be seen in the case of activation of the beta interferon gene, whereby the HMG I(Y) protein facilitates the assembly of functional promoter complexes (7).

The E2F proteins, like many regulatory transcription factors, are modular in structure with separable DNA-binding and transcriptional activation domains. A fusion of the E2F1 activation domain to the GAL4 DNA-binding domain activates transcription in both mammalian cells and *Saccharomyces cerevisiae* (6, 10, 18, 29, 45). Like many transcriptional activation domains, this region of E2F is highly acidic and has been shown to have the capacity to bind to the TATA box-binding protein (16). Likewise, the activation domain of E2F1 can be replaced with the activation domain of herpes simplex virus protein VP16 (26), generating a chimera that can efficiently activate transcription of a promoter containing multimerized E2F-binding sites, but it cannot interact with the Rb protein (26), which appears to interact with the activation domain of E2F1 (5, 10, 16, 18, 19, 29, 45).

In addition to the fact that the E2F family exhibits characteristics of modular transcription factors, it also appears that

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E2F may play a role in transcriptional regulation by altering the architecture of the promoter. We found in this study that the E2F recognition site itself contains an intrinsic DNA bend. We also found that the binding of free E2F to this recognition site results in a DNA bend similar in magnitude to the intrinsic bend but in the opposite orientation. Analysis of a series of E2F1 deletion mutants identified a highly conserved internal portion of E2F1, termed the marked box, as critical for the ability of free E2F1 to reverse the intrinsic bend of the E2F site. Furthermore, we found that the interaction of Rb with E2F1 nullifies its ability to reverse the intrinsic bend of the E2F site. Finally, we also found that DNA bending of the E2F1 promoter, either the intrinsic bend or the E2F1-induced bend, is critical for transcriptional activity of the promoter.

MATERIALS AND METHODS

Construction of phasing analysis vectors. Phasing analysis vectors pTK-401-26 and pTK-401-28 were gifts from Tom Kerppola and have been previously described (30). pBend2 was a gift from Sankar Adhya (32). The phasing analysis series containing the E2F site from the adenovirus E2 promoter was constructed by cloning the hybridized oligonucleotide pairs CTAGATAGTTTTCGCGCTTG and TCGACAAGCGCGAAAACTAT, CTAGATAGTTTTCGCGCTTAAATG and TCGACATTTAAGCGCGAAAACTAT, and CTAGATAGTTTTCGCGCTTAAATTTGAGG and TCGACCTCAAATTTAAGCGCGAAAACTAT into the *Sal*I-*Xba*I cloning sites of pTK-401-26 and pTK-401-28. The phasing analysis series containing the E2F site from the E2F1 promoter (27) was constructed by PCR with plasmid pBEND2 as the template and the oligonucleotide TAGGCGTATCACGAGGCCCT, in combination with oligonucleotides GCGTGTCGACTTTGCGCGGAAATCTAGAGGATCCCTCG, GCGTGTCGACACTTTTTGCCGCGAAATCTAGAGGATCCCTCG, and GCGTGTCGACGGCCACTTTTTGCCGCGAAATCTAGAGGATCCCTCG, to generate three DNA fragments which were then digested with *Eco*RI and *Sal*I and cloned into the *Eco*RI and *Sal*I sites of pTK-401-26 and pTK-401-28.

Electrophoretic mobility shift assays and phasing analysis. Probes corresponding to phasing analysis vectors were prepared by incorporating labeled deoxynucleotides (Klenow) into restriction enzyme-digested *Eco*RI-*Hin*dIII fragments (30). The incubation conditions used for DNA binding have already been described (54). Initial experiments aimed at determining the inherent bend in the E2F1 and E2 promoter sequences utilized standardized conditions (30). Once the inherent DNA bend angles for E2F sites were determined, the unbound probes were used to calibrate the relationship between bend angle and mobility variation for gel conditions more appropriate for high-molecular-weight complexes (54). The *k* constant (30) for our standard gel conditions (54) was estimated to be 0.72. Because of the inherent bend in the naked DNA, it was inappropriate to correct complex mobilities for variations in the probe mobilities. Instead, complex mobilities were normalized to the average mobility of all complexes. Data from at least three individual experiments were then fitted to a cosine function (the phasing function of Kerppola and Curran [30] by using CurveFits 4.27 [John P. Arkins]), and the amplitude of the phasing function was then used to estimate the bend angle (30). The orientation of DNA bending was determined from the minima and maxima of the phasing function, assuming a minor-groove-oriented bend of 54° at the center of the AT tract control bend (30, 33, 48).

Expression vector constructs and protein expression. E2F1 expression plasmids CMV-E2F1, CMV-E2F1$_{89–437}$, and GST-E2F1 have been previously described (5). CMV-DP1 was a gift from Kristian Helin (20). CMV-E2F2$_{85–437}$ and CMV-E2F3$_{132–425}$ were gifts from Jacqueline Lees and have been previously described (37). CMV-E2F1$_{1–417}$ and CMV-E2F1$_{1–283}$ were a gift from David Johnson, and similar constructs have been previously described (17, 18).

GST-E2F1$_{1–283}$ was constructed by cloning an *Eco*RI fragment from pHB44-E2F1$_{1–283}$ (6) into the *Eco*RI site of pGEX-1X. Glutathione *S*-transferase (GST) fusion proteins were expressed in *Escherichia coli* and purified in accordance with previously described procedures (46). Transient overexpression of E2F1, E2F2, E2F3, and DP1 derivatives was achieved by calcium phosphate coprecipitation of plasmid DNA into C33A cells. This method has previously been shown to transiently overexpress E2F1 (and the E2F1 derivatives used in this work) as much as 100-fold above the endogenous cellular E2F (6, 17, 18). C33A cells (obtained from the American Type Culture Collection) were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum. Whole-cell extracts were prepared from C33A cells transfected with appropriate E2F1 and DP1 expression plasmids as previously described (6).

Transfection assays and reporter plasmids. Plasmids CMV-β-GAL, pE2F1-Luc(−242) (27), and 4XE2-CAT (43) have been previously described. Mutant derivatives of pE2F1-Luc(−242) were constructed by subcloning the *Sma*I-*Xba*I fragment of pE2F1-Luc(−242) into M13mp19Rf. Mutants were generated by oligonucleotide-directed mutagenesis (35). *Xho*IA, *Xho*IB, *Xho*IC, and *Xho*IAC mutants were generated by using the following oligonucleotides (5-bp insertions are underlined): *Xho*IA, ATTGTGGCGGCGCTCGGCGGCTCGAGTCGTGGCTCTTTCGCGGCAAA; *Xho*IB, TGGCTCTTTCGCGGCAAAAAGCTCGAGATTTGGCGCGTAAAAGTGGC; *Xho*IC, AAGGATTTGGCGCGTAAAAGTCTCGAGGCCGGGACTTTGCAGGCAGC. Mutant promoter fragments were then cloned back into *Sma*I-*Xba*I vector pGL2, and sequences were confirmed by double-stranded sequencing. Transfection of REF52 cells and assays of the luciferase, chloramphenicol acetyltransferase, and β-galactosidase reporters were done essentially as previously described (27).

RESULTS

DNA containing an E2F recognition site contains an intrinsic DNA bend. To quantitatively analyze DNA bending by the E2F family of transcription factors, we used a method known as phasing analysis, which is based on the phase-dependent interaction between two closely spaced bends in a DNA helix (2, 30, 31). Two series of phasing analysis probes (represented in Fig. 1A) were constructed, each of which contains an intrinsic bend which has a well-characterized orientation and magnitude (33). The intrinsic bend used in our analysis consisted of three phased AT runs which have been shown to bend DNA toward the minor groove at the center of the AT run. Two different E2F binding sites, one from the adenovirus E2 promoter (Ad-E2) and one from the E2F1 promoter (h-E2F1), were placed adjacent to these sites. Phasing analysis of the two different E2F sites containing free probes by using the standardized conditions described by Kerppola and Curran (30) revealed that the E2F sites contained significant intrinsic bends (Fig. 1B). The E2F site from the adenovirus E2 promoter, possessing the central 12-bp sequence TTTCGCGCTTAA, exhibits an intrinsic DNA bend of 25 ± 2° as determined from the amplitude of the phasing function (30). The E2F site from the E2F1 promoter (27), which has the central 12-bp sequence TTTCGCGGCAAA, exhibits a more dramatic intrinsic bend of 40 ± 2° as determined by the amplitude of the phasing function. The orientation of the intrinsic DNA bends for each of the two E2F sites tested appears to be toward the major groove relative to the center of the E2F sites as determined from the maxima and minima of the mobility pattern (30, 31, 55).

E2F binding reverses the intrinsic DNA bend of the E2F site, whereas the Rb protein cancels the E2F effect. Having established that E2F sites exhibit an intrinsic bend, we sought to measure the effects of E2F binding upon the inherent bend. The standardized conditions (30) that were used to characterize the intrinsic DNA bends were not appropriate for analysis of slowly migrating E2F-DNA complexes. We thus used the intrinsic DNA bends within the E2F1 promoter to calibrate more typical E2F gel conditions (54) as described in Materials and Methods. Shown in Fig. 2 are the results of adding a highly purified E2F fraction to the E2F1 promoter series. The first six lanes of Fig. 2A represent the mobilities of naked DNA, whereas the next six lanes show the mobilities of E2F-DNA complexes generated upon the addition of purified E2F. Although the mobility pattern of E2F-DNA complexes is consistent with DNA bending, the shape of the E2F-DNA pattern is reversed relative to that of the naked DNA. Thus, E2F binding reverses the inherent DNA bend and generates a new bend toward the minor groove relative to the center of the E2F site.

The effect of interaction of the Rb protein with E2F on the DNA-bending properties of E2F is shown in the assays in the last six lanes of Fig. 2A. The Rb-E2F-DNA complex was obtained by using extracts of U937 cells in which the predominant form of E2F is the Rb complex. As in the case of free DNA, the mobility pattern of the Rb-E2F-DNA complex is consistent with a bend in the direction of the major groove relative to the center of the E2F site. Thus, the interaction of Rb with E2F

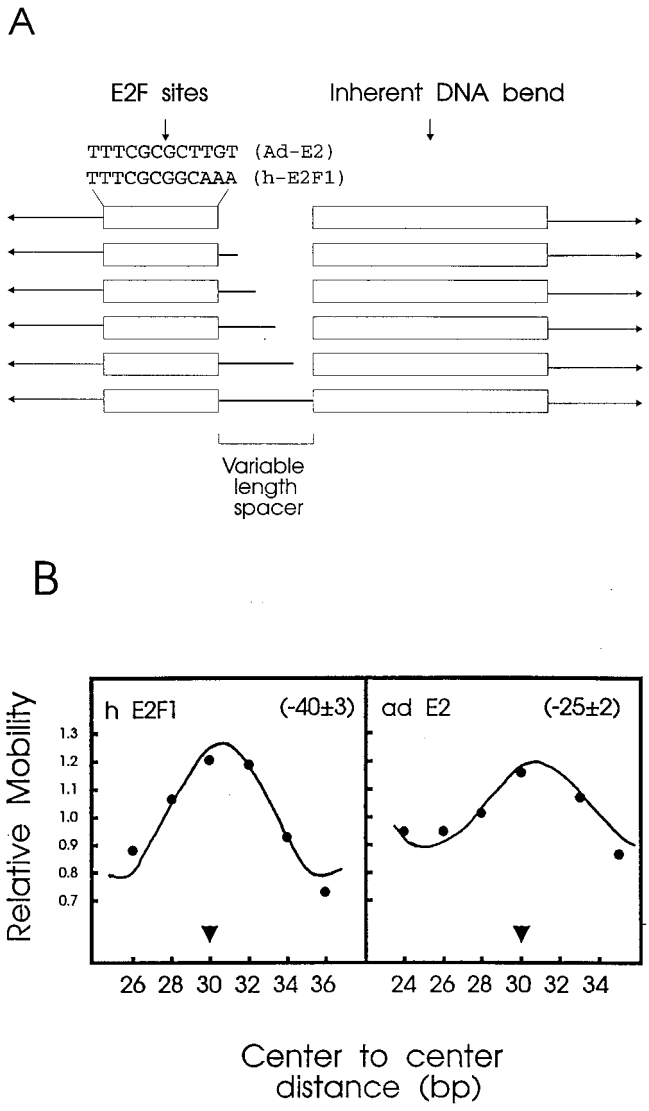

FIG. 1. E2F sites contain an intrinsic bend toward the major groove relative to the center of the E2F site. (A) Two series of constructs were utilized for phasing analysis. The first contained an E2F site from the adenovirus E2 promoter (Ad-E2) centered at 24, 26, 28, 30, 33, and 35 bp from the center of the inherent DNA bend. The second series contained an E2F site from the E2F1 promoter (h-E2F1) centered at 26, 28, 30, 32, 34, and 36 bp away from the center of the inherent DNA bend. Each promoter series was prepared by restricting plasmids with *Hin*dIII and *Eco*RI, purifying the 350- to 360-bp fragments by electrophoresis, and then using a Klenow filling reaction to label the fragments. (B) Phasing analysis reveals inherent bending associated with E2F sites. The two series of phasing analysis vectors depicted in panel A were analyzed by using standardized gel conditions (30). The relative mobilities of free DNA probes are plotted versus the center-to-center distance between the E2F site center and the center of the inherent bend. The points (which are larger than the standard deviations in mobility observed in three separate experiments) are connected by the best fit of a cosine function. The magnitudes of the intrinsic bends (presented in the upper right-hand corner of each box arbitrarily as negative numbers) were determined from the amplitude of the phasing function as previously described (30). The phasing function maxima are highlighted with arrowheads.

neutralizes the ability of E2F to reverse the inherent DNA bend.

The relative bend angles of the E2F-DNA and Rb-E2F-DNA complexes were analyzed quantitatively as shown in Fig. 2B. The intrinsic bend of the E2F1 promoter is $-40 \pm 2°$ oriented toward the major groove relative to the center of the

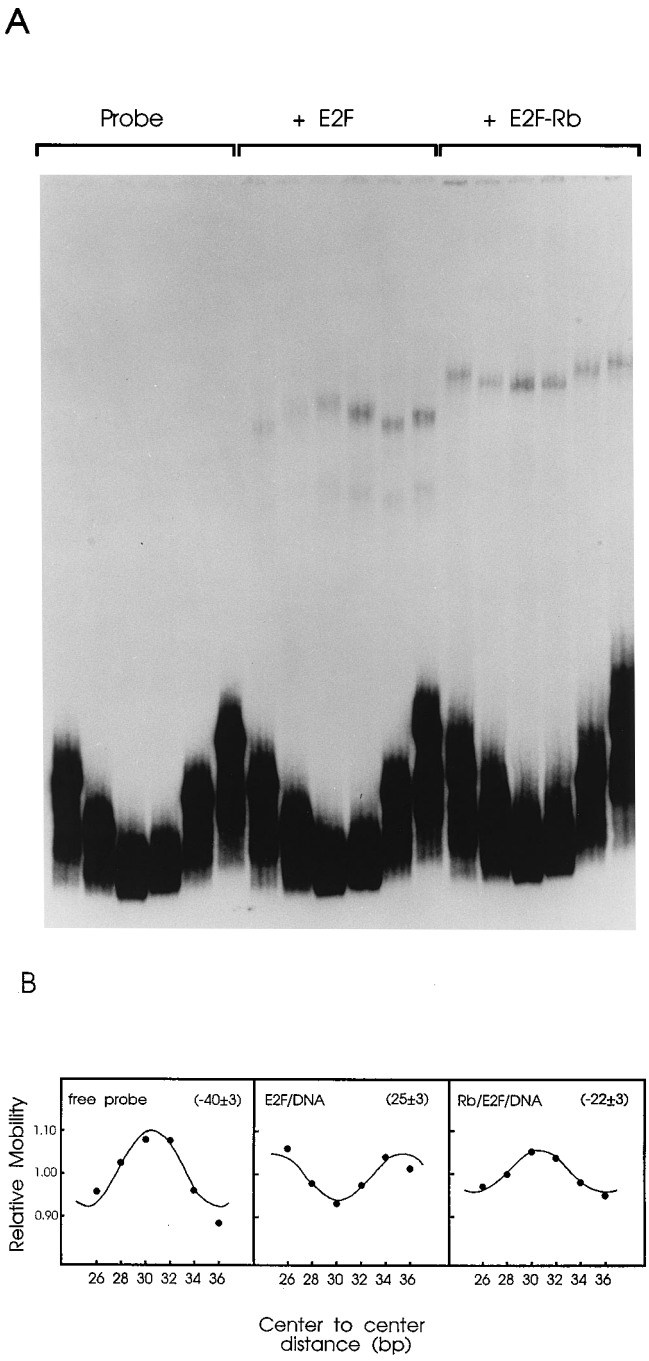

FIG. 2. E2F binding reverses the intrinsic DNA bend of the E2F site, but interaction with the Rb protein cancels the E2F effect. (A) E2F DNA-binding assays in a phasing analysis experiment using the E2F site from the E2F1 promoter (27) and standard E2F gel shift conditions (54). The first six lanes represent the naked DNA; the intrinsic bend in the E2F site DNA is apparent. The next six lanes represent the addition of a highly purified E2F fraction from HeLa cells (54). The final six lanes represent an Rb-E2F-DNA complex obtained from U937 extracts (antibody supershift experiments authenticating this complex are not shown). (B) The relative mobilities from four different gel shift experiments such as the one shown in panel A were averaged, fitted to a cosine curve, and plotted as a function of the center-to-center distance between the E2F site and the center of the inherent DNA bend (data were analyzed as described in Materials and Methods). The bend angles shown were estimated from the amplitude of the phasing function defined by Kerppola and Curran (30). Negative values arbitrarily refer to the intrinsic DNA bend, which is toward the major groove relative to the center of the E2F binding sites, whereas positive values refer to the protein-induced bend. Linear DNA would have a bend angle of 0°.

E2F site (this intrinsic bend was arbitrarily assigned a negative value). The E2F-DNA complex is a reversal of the intrinsic bend, with an estimated bend angle of 25 ± 3° oriented toward the minor groove relative to the center of the E2F site (the protein-induced bend was assigned a positive value). E2F binding to the E2F site thus induces a net change in direction of approximately 65° relative to naked DNA. The estimated bend angle of the Rb-E2F-DNA complex is −23 ± 3° oriented toward the major groove relative to the center of the E2F site, thus indicating that the interaction with Rb substantially nullifies the ability of E2F to reverse the intrinsic DNA bend of the E2F site. Because the E2F site is intrinsically bent, it is formally possible that some of the effect measured in phasing analysis is due not to a protein-directed bend but rather to protein-directed unwinding of the DNA helix between the two intrinsic DNA bends. However, we expect that bending is a primary component of the effect that we observed with phasing analysis, since bending was also detected with circular permutation analysis, which should not detect DNA unwinding (23).

The E2F1, E2F2, and E2F3 products can bend the E2F1 promoter DNA toward the minor groove. The total E2F DNA-binding activity as purified from nuclear extracts represents a family of E2F activities (22). In addition, most of the cellular E2F activity is represented as heterodimeric complexes (1, 20, 34) including one member from the E2F family (3, 13, 25, 37) and one member from the DP1 family (14). Given this complexity, we assayed individual members of the E2F family for their effect on DNA bending. Cloned E2F1, E2F2, and E2F3 gene products, in combination with the heterodimeric partner DP1, were overexpressed in C33A cells, and the resulting heterodimers were assayed for their DNA-bending properties. As shown in Fig. 3A, each of the E2F-DP1 combinations yielded a mobility pattern with the same shape as the total cellular E2F activity assayed as shown in Fig. 2. Thus, each of these cloned E2F products reverses the intrinsic bend to generate a bend toward the minor groove relative to the center of the E2F site. Quantitative analysis, presented in Fig. 3B, revealed that the E2F1, E2F2, and E2F3 gene products, in combination with DP1, generate bends similar in magnitude (16 ± 3, 11 ± 3, and 14 ± 4°, respectively).

Identification of E2F1 residues required for DNA bending. The interaction of the E2F family members with Rb is mediated, at least in part, by the C-terminal portion of each protein, sequences which are also critical for the transcriptional activation capacity of the protein (5, 10, 16, 18, 19, 29, 37, 45). Since the interaction of the Rb protein nullifies the ability of E2F to bend DNA, it was possible that the activation domain of E2F could contribute to DNA bending even though it is known not to be required for binding to DNA (19, 29, 45). A series of E2F1 deletion derivatives (Fig. 4A) were transiently overexpressed in C33A cells in combination with a full-length DP1 clone, and then extracts from the transfected cells were assayed for E2F activity by gel retardation. Deletion of the N-terminal 88 amino acids of E2F1 did not alter the DNA-bending property compared with that of the wild-type E2F1 protein (Fig. 4C). Similarly, the $E2F1_{1–417}$ mutant, in which part of the transactivation domain was removed, was not significantly diminished in DNA-bending activity relative to the wild-type E2F1 heterodimer with DP1. Thus, the formally defined Rb-binding domain is not critical to DNA bending. However, further deletion of amino acids 284 to 437, creating $E2F1_{1–283}$, which partially eliminated a region termed the marked box (37), profoundly reduced DNA bending by the resulting DP1 heterodimer. It thus appears that amino acid sequences representing the highly conserved marked-box domain, which are not required for DNA binding, do contribute to the DNA-bending activity of E2F1.

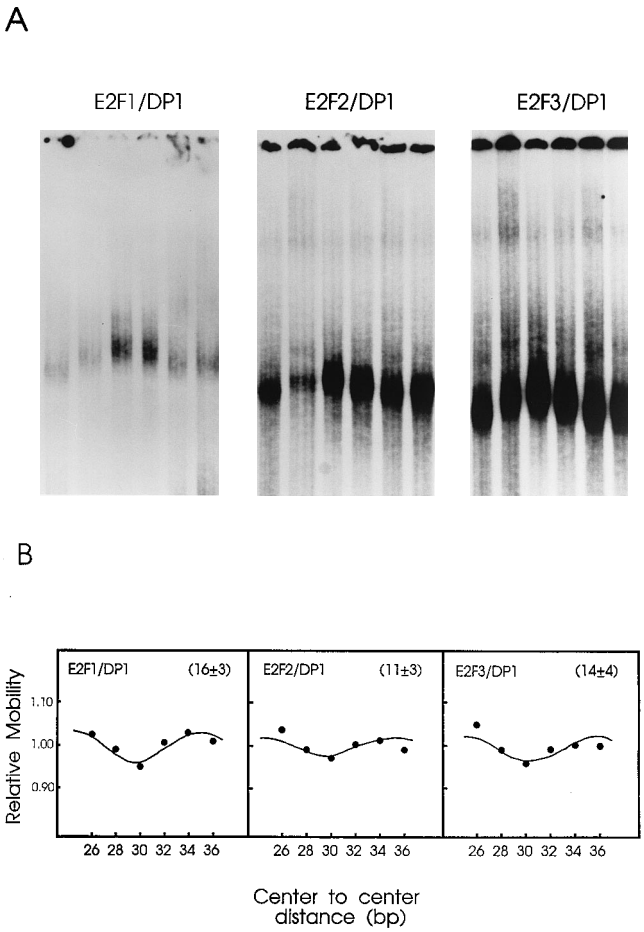

FIG. 3. Phasing analysis of the cloned E2F products reveals that they each bend the DNA toward the minor groove relative to the center of the protein half site. (A) The E2F1, E2F2, and E2F3 products, in combination with DP1, were transiently overexpressed in C33A cells as previously described (6, 20). Transiently expressed E2F-DP1 combinations were typically overexpressed 100-fold above the level of endogenous E2F DNA-binding activity. The E2F and DP1 expression vectors have been described previously: E2F1, reference 5; E2F2 (amino acids 85 to 437) and E2F3 (amino acids 132 to 425), reference 37; DP1, reference 20. The probe series and gel conditions were the same as those described in the legend to Fig. 2A. (B) Quantitative analysis of E2F1-DP1, E2F2-DP1, and E2F3-DP1 combinations. The relative mobilities of E2F-DP1-DNA complexes averaged from four different experiments are plotted versus center-to-center distance. The bend angles estimated from the amplitude of the phasing function are shown in the upper right corners of the graphs.

We next examined the relative contribution of the E2F1 activation domain (amino acid residues 358 to 437) and the marked-box domain (residues 251 to 317), in the context of E2F1 homodimers, to exclude the contribution of the full-length DP1 partner which appears to contribute substantially to DNA bending. Although it is unlikely that E2F1 homodimers are important in vivo, it has been shown that the GST-E2F1 protein can bind DNA as a homodimer (25). For this analysis, GST fusions of the full-length E2F1 protein, a truncated version lacking the activation domain ($E2F1_{1–358}$), and a third construct lacking the marked-box and activation domains ($E2F1_{1–283}$) were assayed as homodimers for DNA bending. The analyses depicted in Fig. 4C reveal that the full-length GST-E2F1 homodimer was able to reverse the intrinsic DNA bend to generate a protein-induced bend of approximately 9 ±

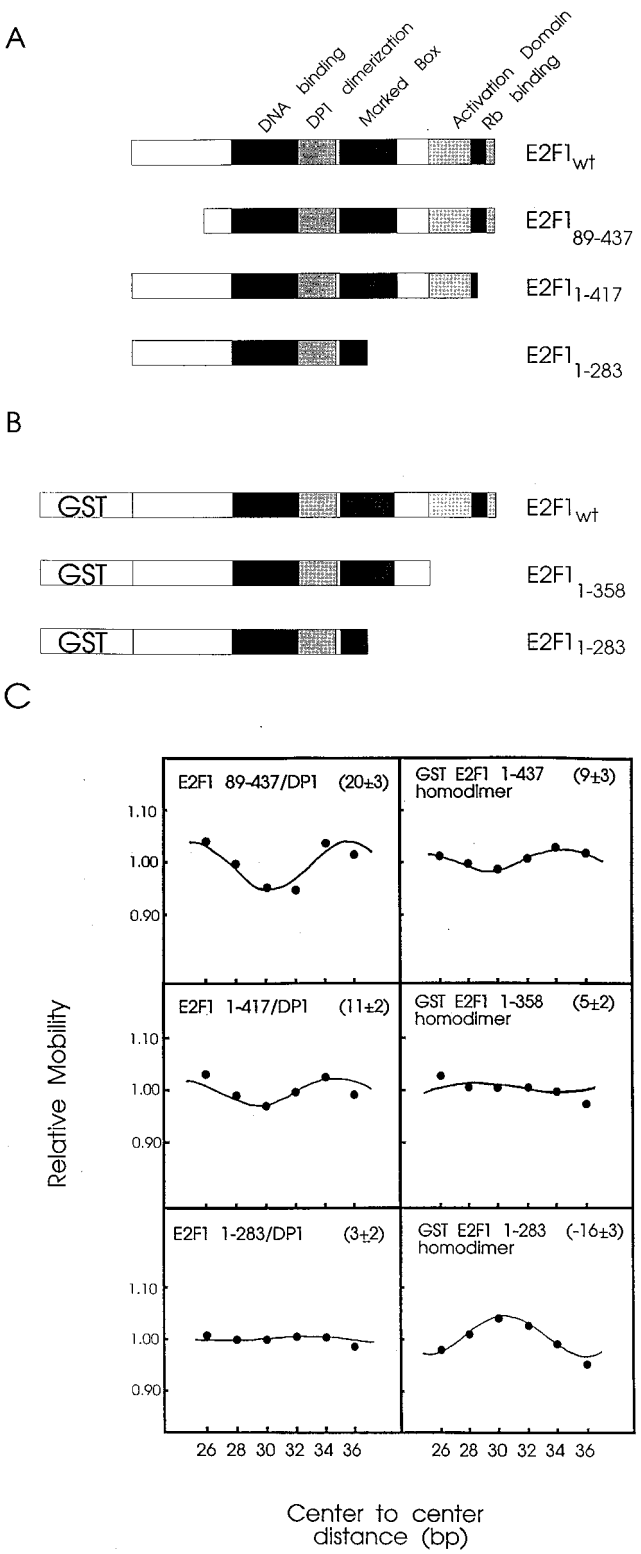

FIG. 4. The marked-box domain of E2F1 contributes to DNA bending. (A) Schematic representation of E2F1 deletion mutants characterized in bending assays. This series of E2F1 constructs were overexpressed in C33A cells in combination with full-length DP1 as previously described (6, 20). Previously defined structural domains are highlighted. wt, wild type. (B) Schematic representation of E2F1 deletion mutants expressed and purified from *E. coli* as GST fusion proteins. These proteins bind DNA as homodimers (25). (C) Phasing analysis employing the E2F1 derivatives described in panels A and B indicates a primary role of the E2F1 marked box and a contributory role for the E2F1 activation domain in DNA bending. The data shown are from at least three independent experiments and were analyzed as described in Materials and Methods. Approximately 10 ng of each GST protein was included in each reaction mixture.

3° toward the minor groove relative to the center of the E2F site (Fig. 4C). Deletion of the E2F1 activation domain (as occurs in the $E2F1_{1-358}$ mutant) did not have a statistically significant effect on DNA bending relative to the full-length construct (9 ± 3 compared with 5 ± 3°). The contribution of the E2F sequences referred to as the marked box (37) became evident when the $E2F1_{1-283}$ homodimer was analyzed for DNA bending. Although this protein bound specifically to the DNA, it was unable to reverse the direction of the intrinsic DNA bend. The net bend in the GST-$E2F1_{1-283}$-DNA complex (although diminished in magnitude by the bound protein at −40 ± 2° compared with −16 ± 2°) remains in the direction of the major groove. The results obtained with these bacterially produced proteins highlight the role of the E2F1 marked-box region in DNA bending.

A bent DNA structure contributes to activation of the E2F1 promoter. The unusual structure that the E2F elements impart to the promoter, together with the fact that E2F binding to these sites has a dramatic effect on this structure, strongly suggests a role for DNA structure in E2F-dependent transcription control. We used the E2F1 promoter as a model to examine the contribution of the intrinsic DNA bend and the E2F-mediated DNA bending in transcriptional regulation. The *cis*-acting elements of the E2F1 promoter, which are critical for cell cycle-regulated transcription, are contained within a DNA region approximately 200 bp long which is highly conserved between the mouse and human genomes. Previous work has shown that the wild-type E2F1 promoter is repressed in serum-starved cells, dependent on the two E2F sites, and is then activated when the cells are induced to proliferate by addition of serum (21, 27, 41). A derivative of the E2F1 promoter lacking the E2F sites was not efficiently repressed in serum-starved cells and was not further activated upon serum addition, consistent with a role for E2F-Rb repression of transcription through these sites. If DNA bending is an important aspect of promoter regulation, one would predict that 5-bp insertions (one-half of a helical turn) adjacent to or between the two E2F sites would diminish the architectural role of E2F and might alter some regulatory aspect of the E2F1 promoter.

Figure 5A highlights the structure of the E2F1 promoter mutants which were generated. Mutant A had a 5-bp insertion upstream of the E2F sites, mutant B had a 5-bp insertion between the two E2F sites, and mutant C had a 5-bp insertion between the E2F sites and the major transcription start site. Mutant AC had 5-bp insertions both upstream and downstream of the E2F sites. The activity of the mutant E2F1 promoters in quiescent and stimulated REF52 cells was then compared with those of the wild-type E2F1 promoter and an E2F1 promoter in which the two E2F sites were mutated (27). This mutant promoter does not bind E2F, but the intrinsic bend is not altered from that of the wild-type sequence (data not shown). As shown in Fig. 5B, the mutations which altered the phase of the E2F1 promoter did not significantly alter the low basal level of the E2F1 promoter in serum-starved cells. Thus, the E2F-Rb repression of the E2F1 promoter does not appear to be dependent on a specific promoter structure. In contrast, the 5-bp insertions did diminish the activation of the E2F1 promoter following serum stimulation. These promoter mutants had similarly diminished responses to exogenous

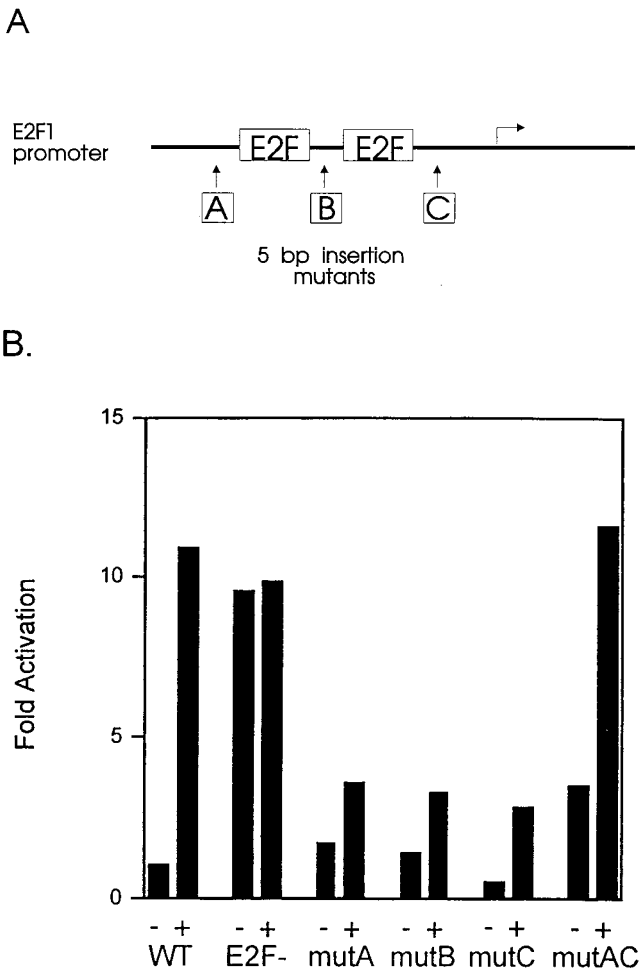

FIG. 5. Architecture within the E2F1 promoter is important for E2F-mediated activation of transcription. (A) Depicted is a 200-bp region of the E2F1 promoter that is sufficient for cell cycle regulation of the E2F1 gene and is highly conserved between the mouse and human promoters (21, 27, 41). The bent arrow represents the major transcription start site, and the boxes represent the two E2F-binding sites of the promoter. Mutations of the E2F promoter which were generated are highlighted below the wild type, and the vertical arrows represent 5-bp insertions which also generated *Xho*I sites. (B) Phase-changing mutations do not diminish repression of the E2F1 promoter in serum-starved REF52 cells but do diminish activation following serum induction. REF52 cells were transfected with 5 μg of the luciferase reporters (as indicated) along with 3 μg of a cytomegalovirus-driven β-galactosidase internal control and 12 μg of carrier DNA as previously described (27). Following serum starvation for 48 h, some of the cells were induced to proliferate by addition of 20% fetal calf serum. Cells were harvested 24 h later, and luciferase and β-galactosidase levels were determined in extracts of the cells. Values for luciferase assays are expressed as fold activation and are averages of two independent transfections. In each case, luciferase activity was corrected by using the β-galactosidase internal control. The E2F-negative mutant has been previously described (27). Activity in serum-starved and serum-stimulated cells is indicated by minus and plus signs, respectively. WT, wild type.

E2F1 expression (data not shown). Unlike the single insertions, the insertion of two 5-bp sequences on either side of the E2F sites (Fig. 5B, mutAC) preserved a high level of activation in serum-stimulated cells. The observation that single 5-bp insertions diminished activation whereas two 5-bp insertions preserved activation suggests that the structure of the E2F1 promoter is important for activity.

To further address the contribution of DNA bending to promoter activation, we assayed a series of E2F1 mutant proteins for activation of the promoter. These include wild-type E2F1; $E2F1_{FS409}$, which bends DNA but lacks an activation domain; and $E2F1_{1–283}$, which lacks an activation domain and generates straight DNA upon binding. These E2F1 derivatives were characterized for the ability to activate the E2F1 promoter, where we anticipated DNA bending to play a role, as well as an artificial construct in which an E2F site is reiterated eight times adjacent to the TATA box of the adenovirus E2 promoter (4XE2-CAT) (43). Because 4XE2-CAT contains no other recognized transcription factor-binding sites, it is likely that E2F-mediated transcription activation of this promoter involves direct interactions of the bound E2F factors with proteins of the basal machinery, including TFIID.

Expression of wild-type E2F1 together with DP1 activated both promoters (Fig. 6). E2F1 frameshift mutant $E2F1_{FS409}$, which lacks the acidic activation domain and did not interact with the TATA-binding protein in pull-down assays (data not shown), failed to activate the 4XE2-CAT reporter as expected on the basis of removal of the activation domain. In sharp contrast, the $E2F1_{FS409}$ mutant, which does retain the ability to bend DNA, as shown in Fig. 6B, also retained the capacity to activate the E2F1 promoter. The activation seen in this assay is likely the consequence of displacement of the inhibitory E2F-Rb complex from the E2F-binding sites. Since this E2F1 protein still bends the DNA, the bent structure of the promoter is retained. In contrast, the $E2F1_{1–283}$ mutant, which lacks the marked-box domain in addition to the activation domain and has significantly reduced DNA-bending capacity (Fig. 4C), did not activate either the 4XE2 promoter or the E2F1 promoter.

From these results, we conclude that the structure of the E2F1 promoter does affect the transcriptional activity of the promoter. Five-base-pair substitutions in and around the E2F sites likely reduce promoter activity because they rotate the DNA helix 180°, thus adversely affecting the interactions of upstream factors such as SP1 with the basal-complex factors. The absence of E2F binding to the promoter (with the E2F site mutant) still allows promoter activation, which is likely the consequence of relief of repression imposed by E2F-Rb binding. This repression can also be reversed by competition with an excess of free E2F. This reversal can be accomplished by both wild-type E2F1 and an activation domain mutant, since they both would displace the Rb complex. Both of these proteins are able to bend the DNA, and although the bend is in the direction opposite to the naked DNA, the resulting bend angle is nearly equivalent and thus upstream sites and basal-complex sites would still be brought into juxtaposition in the same plane. In contrast, the binding of the E2F mutant ($E2F1_{1–283}$) that changes this bend angle, generating a nearly linear DNA structure, does not allow activation even though it would also displace the E2F-Rb repressor. Thus, activation of the E2F1 promoter directly correlates with the structure of the promoter involving the E2F recognition sites.

DISCUSSION

Rapid advances have been made over the past several years in the understanding of mechanisms of transcriptional regulation. This includes the identification of large numbers of transcriptional regulatory proteins that participate in the assembly of the basal-promoter complex, as well as the activating proteins that bind upstream sequences and influence the frequency of transcription initiation. Although most attention has focused on the function of activation domains and the role of interactions of these domains with components of the basal-promoter complex, a number of recent studies have shown that

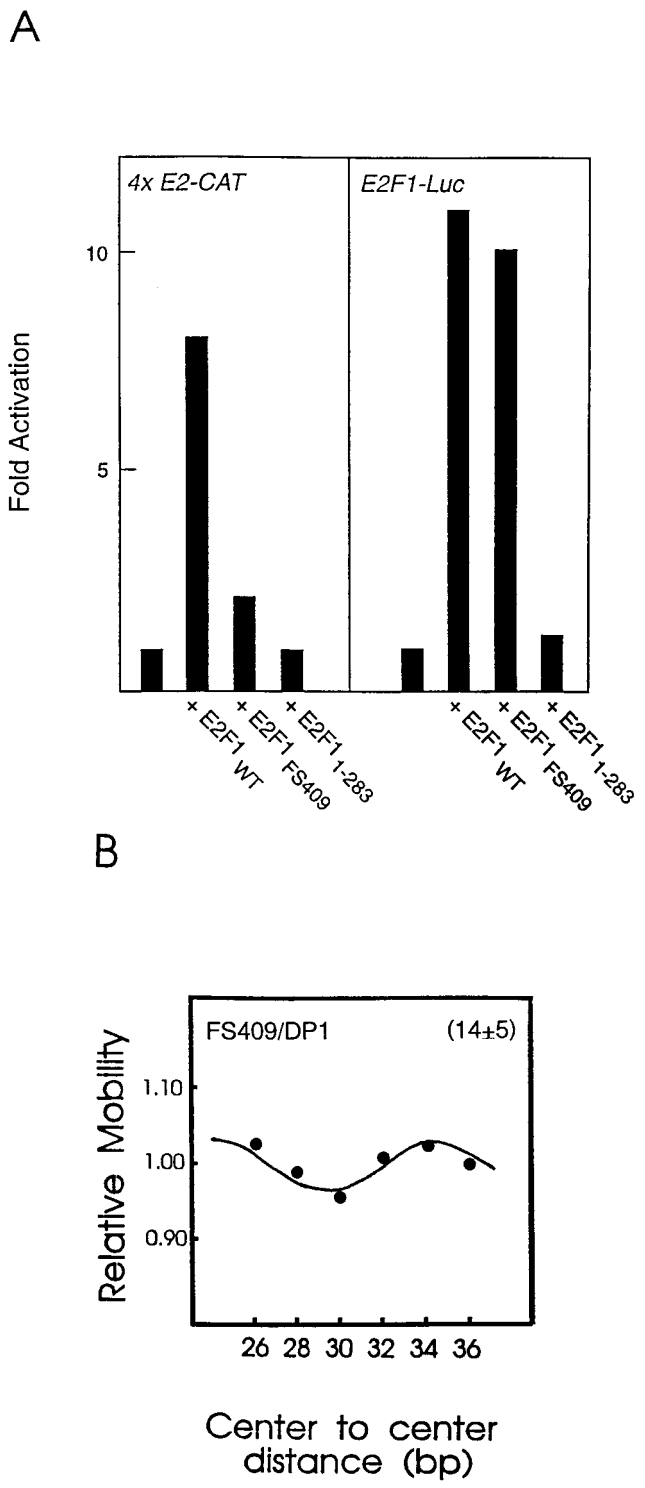

FIG. 6. Activation of the E2F1 promoter by E2F1 mutant proteins supports a role for DNA bending in E2F-mediated transcriptional activation. (A) Differential activation of 4XE2-CAT and E2F1-Luc by various E2F1 derivatives. Cycling REF52 cells were transfected with 5 μg of the wild-type (WT) E2F1-Luc reporter, 5 μg of the 4XE2-CAT reporter, 3 μg of a cytomegalovirus-driven β-galactosidase internal control, and 6 μg of carrier DNA as previously described (27). Transfections included 500 ng each of an E2F1-expressing plasmid and a DP1-expressing vector or an equal amount of an empty vector. Cells were harvested 48 h following transfection. The data are averages of two independent transfection assays. (B) Phasing analysis reveals that FS409 bends DNA as well as wild-type E2F1 does. FS409 was overexpressed in combination with DP1 and assayed as described in the legend to Fig. 3.

the architecture of the promoter is important in allowing these interactions to form. In some cases, this involves the action of proteins that alter the inherent structure of the promoter DNA without themselves playing a direct role in activation (7, 49, 52). In other instances, transcription factors that possess activation domains, which likely participate in direct interactions with the transcriptional machinery, also have the capacity to alter the promoter structure to facilitate the interactions (40). The experiments we present here demonstrate that the E2F transcription factor family represents an example of this latter class of proteins.

E2F as an element with DNA-bending activity. The previous work of Huber and colleagues investigated DNA bending mediated by E2F, as well as the effect of Rb on an E2F-mediated bend (23). These experiments estimated that free E2F induced a DNA flexure angle of 125° and that the interaction of Rb with E2F reduced the bend angle to 80°. These investigators did not report an intrinsic bend in the DNA. The discrepancy of results clearly lies in the choice of method used to measure bending, since the experiments performed by Huber and colleagues utilized circular permutation analysis, which does not allow accurate estimation of bend direction or magnitude and is sensitive to the shape of the proteins being analyzed (30, 31).

Through assays of various E2F1 mutants, the experiments we present here demonstrate that an internal portion of the E2F1 protein, the so-called marked-box domain (37), is critical for its ability to bend DNA and reverse the intrinsic DNA bend of the E2F recognition site. The marked box of E2F1 has been implicated as important for interactions with the adenovirus E4 protein, as well as the Rb protein (8, 17, 42). Whether these properties relate to the mechanisms by which the E2F marked-box domain might contribute to DNA bending and how the interaction of Rb nullifies the bending capacity of E2F1 remain unclear.

The best-studied example of protein-induced DNA bending by a protein structurally related to E2F is the B-Zip-containing Fos-Jun heterodimer (30, 31). Computer modeling of the Fos-Jun-DNA interaction predicts that the Fos protein induces a bend away from the dimer interface. In the Fos-Jun-DNA complex, it is expected that the Fos basic region forms a continuous alpha helix which causes the major groove to bend to support charge interactions between the phosphates of the DNA and basic side chains of the protein. By analogy, we predict that upon binding to the intrinsically bent DNA, basic helices of E2F likely fill the major groove and rather than break the protein helix to continue to form a tract along the major groove, the DNA itself bends to accommodate a continuous protein alpha helix. This model is consistent with the footprints of E2F-DNA complexes, since E2F protects a relatively large area of the DNA, as might be expected if extensive interactions between the protein alpha helix and the major groove of the DNA were occurring (29, 54). In addition, the fact that the conformation of the E2F-Rb-DNA complex is very close to the state of the intrinsic bend of the naked DNA suggests that the 10- to 15-fold longer half-life of the Rb complex versus the E2F-DNA complex (23) may reflect the energy required to reverse the intrinsic bend, making the complex less stable than the E2F-Rb-DNA complex, where no energy would be required to alter the DNA structure. Since a role of the E2F-Rb complex is to inhibit transcription from otherwise active promoters (27, 53), the greater stability of the Rb-containing complex may afford tighter control of transcription.

Relationship between transcription control by E2F, E2F-Rb, and promoter architecture. Our experiments suggest that in the context of the E2F1 promoter, a DNA bend in either

direction, whether the intrinsic bend dictated by the DNA sequence or the bend induced by bound E2F, contributes to transcription activation, presumably by bringing proteins closer together that are bound to matching faces of the DNA helix. We suspect that the intrinsic bend and the E2F-mediated bend, which create DNA structures with similar bend angles, may facilitate the ability of upstream activators such as SP1 to make appropriate contacts with the basal transcription machinery, including the TATA-binding-protein-associated factor components of transcription factor TFIID (4), as a result of physical juxtaposition. We believe that the various circumstances that reduce the transcriptional activity of the promoter provide compelling evidence that DNA bending is important for promoter activity. The 5-bp substitutions in and around the E2F sites likely reduce promoter activity because they rotate the DNA helix 180°, thus adversely affecting the interactions of upstream factors such as SP1 with the basal-complex factors. Although the binding of the wild-type E2F1 protein reverses the DNA bend, the spatial relationship of upstream and downstream components is maintained in the same plane and the promoter is active. This is highlighted by the observation that an E2F1 mutant that lacks the activation domain but retains the ability to bend the DNA ($E2F1_{FS409}$) can still activate the E2F1 promoter, whereas an E2F1 mutant that straightens the promoter DNA upon binding ($E2F1_{1-283}$), and thus disrupts the spatial relationship of upstream sites to the basal complex, does not activate the promoter. For instance, if one imagines the DNA lying on a flat surface with two transcriptional complexes bound on top of the DNA, then a bend in either direction within the plane of the surface will bring the complexes closer together. This result strongly suggests that the alteration of the structure of the DNA has a significant impact on the transcriptional activity of the promoter.

On the basis of these observations, we believe it is likely that the activity of the E2F1 promoter, and possibly that of any promoter containing E2F recognition sites, is influenced by the inherent structure dictated by the sequence of these elements. Although the E2F-dependent activation of the E2F1 promoter during a cell growth response may be relatively independent of the activation domain, we suspect that the ability of the protein to displace the repressing E2F-Rb complex but still maintain a promoter structure that facilitates interactions of other promoter-binding factors is critically important to the activation of the promoter.

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REFERENCES

1. **Bandara, L. R., V. M. Buck, M. Zamanian, L. H. Johnston, and N. B. La Thangue.** 1993. Functional synergy between DP-1 and E2F-1 in the cell cycle-regulating transcription factor DRTF1-E2F. EMBO J. **12:**4317–4324.
2. **Bazett-Jones, D. P., B. Leblanc, M. Herfort, and T. Moss.** 1994. Short-range DNA looping by the *Xenopus* HMG-Box transcription factor, xUBF. Science **264:**1134–1137.
3. **Beijersbergen, R. L., R. M. Kerkhoven, L. Zhu, L. Carlee, P. M. Voorhoeve, and R. Bernards.** 1994. E2F-4, a new member of the E2F gene family, has oncogenic activity and associates with p107 in vivo. Genes Dev. **8:**2680–2690.
4. **Chen, J.-L., L. D. Attardi, C. P. Verrijzer, K. Yokomori, and R. Tjian.** 1994. Assembly of recombinant TFIID reveals differential coactivator requirements for distinct transcriptional activators. Cell **79:**93–105.
5. **Cress, W. D., D. G. Johnson, and J. R. Nevins.** 1993. A genetic analysis of the E2F1 gene distinguishes regulation by Rb, p107, and adenovirus E4. Mol. Cell. Biol. **13:**6314–6325.
6. **Cress, W. D., and J. R. Nevins.** 1994. Interacting domains of E2F1, DP1, and the adenovirus E4 protein. J. Virol. **68:**4212–4219.
7. **Du, W., D. Thanos, and T. Maniatis.** 1993. Mechanisms of transcriptional synergism between distinct virus-inducible enhancer elements. Cell **74:**887–898.
8. **Fagan, R., K. J. Flint, and N. Jones.** 1994. Phosphorylation of E2F-1 modulates its interaction with the retinoblastoma gene product and the adenovirus E4 19 kDa protein. Cell **78:**799–811.
9. **Fisher, D. E., L. A. Parent, and P. A. Sharp.** 1992. Myc/Max and other helix-loop-helix/leucine zipper proteins bend DNA toward the minor groove. Proc. Natl. Acad. Sci. USA **89:**11779–11783.
10. **Flemington, E. K., S. H. Speck, and W. G. Kaelin, Jr.** 1993. E2F-1-mediated transactivation is inhibited by complex formation with the retinoblastoma susceptibility gene product. Proc. Natl. Acad. Sci. USA **90:**6914–6918.
11. **Giese, K., A. Amsterdam, and R. Grosschedl.** 1991. DNA binding properties of the HMG domain of the lymphoid-specific transcriptional regulator LEF-1. Genes Dev. **5:**2567–2578.
12. **Giese, K., J. Cox, and R. Grosschedl.** 1992. The HMG domain of lymphoid enhancer factor 1 bends DNA and facilitates assembly of functional nucleoprotein structures. Cell **69:**185–195.
13. **Ginsberg, D., G. Vairo, T. Chittenden, Z. Xiao, G. Xu, K. L. Wydner, J. A. DeCaprio, J. B. Lawrence, and D. Livingston.** 1994. E2F-4, a new member of the E2F transcription factor family, interacts with p107. Genes Dev. **8:**2665–2679.
14. **Girling, R., J. F. Partridge, L. R. Bandara, N. Burden, N. F. Totty, J. J. Hsuan, and N. B. La Thangue.** 1993. A new component of the transcription factor DRTF1/E2F. Nature (London) **362:**83–87.
15. **Gustafson, T. A., A. Taylor, and L. Kedes.** 1989. DNA bending is induced by a transcription factor that interacts with the human c-fos and α-actin promoters. Proc. Natl. Acad. Sci. USA **86:**2162–2166.
16. **Hagemeier, C., A. Cook, and T. Kouzarides.** 1993. The retinoblastoma protein binds E2F residues required for activation *in vivo* and TBP binding *in vitro*. Nucleic Acids Res. **21:**4998–5004.
17. **Helin, K., and E. Harlow.** 1994. Heterodimerization of the transcription factors E2F-1 and DP-1 is required for binding to the adenovirus E4 (ORF6/7) protein. J. Virol. **68:**5027–5035.
18. **Helin, K., E. Harlow, and A. Fattaey.** 1993. Inhibition of E2F-1 transactivation by direct binding of the retinoblastoma protein. Mol. Cell. Biol. **13:**6501–6508.
19. **Helin, K., J. A. Lees, N. Dyson, E. Harlow, and A. Fattaey.** 1992. A cDNA encoding a pRB-binding protein with properties of the transcription factor E2F. Cell **70:**337–350.
20. **Helin, K., C.-L. Wu, A. R. Fattaey, J. A. Lees, B. D. Dynlacht, C. Ngwu, and E. Harlow.** 1993. Heterodimerization of the transcription factors E2F-1 and DP-1 leads to cooperative trans-activation. Genes Dev. **7:**1850–1861.
21. **Hsiao, K., 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.
22. **Huber, H. E., G. Edwards, P. J. Goodhart, D. R. Patrick, P. S. Huang, M. Ivey Hoyle, S. F. Barnett, A. Oliff, and D. C. Heimbrook.** 1993. Transcription factor E2F binds DNA as a heterodimer. Proc. Natl. Acad. Sci. USA **90:**3525–3529.
23. **Huber, H. E., P. J. Goodhart, and P. S. Huang.** 1994. Retinoblastoma protein reverses DNA bending by transcription factor E2F. J. Biol. Chem. **269:**6999–7005.
24. **Ingles, C. J., M. Shales, W. D. Cress, S. J. Triezenberg, and J. Greenblatt.** 1991. Reduced binding of TFIID to transcriptionally compromised mutants of VP16. Nature (London) **351:**588–590.
25. **Ivey-Hoyle, M., R. Conroy, H. E. Huber, P. J. Goodhart, A. Oliff, and D. C. Heimbrook.** 1993. Cloning and characterization of E2F-2, a novel protein with the biochemical properties of transcription factor E2F. Mol. Cell. Biol. **13:**7802–7812.
26. **Johnson, D. G., W. D. Cress, L. Jakoi, and J. R. Nevins.** 1994. Oncogenic capacity of the E2F1 gene. Proc. Natl. Acad. Sci. USA **91:**12823–12827.
27. **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.
28. **Johnson, P. F., and S. L. McKnight.** 1989. Eukaryotic transcriptional regulatory proteins. Annu. Rev. Biochem. **58:**799–839.
29. **Kaelin, W. G., W. Krek, W. R. Sellers, J. A. DeCaprio, F. Ajchenbaum, C. S. Fuchs, T. Chittenden, Y. Li, P. J. Farnham, M. A. Blanar, et al.** 1992. Expression cloning of a cDNA encoding a retinoblastoma-binding protein with E2F-like properties. Cell **70:**351–364.
30. **Kerppola, T. K., and T. Curran.** 1991. DNA bending by Fos and Jun: the flexible hinge model. Science **2:**1210–1214.
31. **Kerppola, T. K., and T. Curran.** 1991. Fos-Jun heterodimers and Jun homodimers bend DNA in opposite orientations: implications for transcription factor cooperativity. Cell **66:**317–326.
32. **Kim, J., C. Zwieb, C. Wu, and S. Adhya.** 1989. Bending of DNA by gene-

regulatory proteins: construction and use of a DNA bending vector. Gene **85:** 15–23.
33. **Koo, H., H. Wu, and D. M. Crothers.** 1986. DNA bending at adenine-thymine tracts. Nature (London) **320:**501–506.
34. **Krek, W., D. M. Livingston, and S. Shirodkar.** 1993. Binding to DNA and the retinoblastoma gene product promoted by complex formation of different E2F family members. Science **262:**1557–1560.
35. **Kunkel, T. A.** 1985. Rapid and efficient site-specific mutagenesis without phenotypic selection. Proc. Natl. Acad. Sci. USA **82:**488–490.
36. **Landy, A.** 1989. Dynamic, structural, and regulatory aspects of lambda site-specific recombination. Annu. Rev. Biochem. **58:**913–949.
37. **Lees, J. A., M. Saito, M. Valentine, T. Look, E. Harlow, N. Dyson, and K. Helin.** 1993. The retinoblastoma protein binds to a family of E2F transcription factors. Mol. Cell. Biol. **13:**7813–7825.
38. **Lin, Y. S., I. Ha, E. Maldonado, D. Reinberg, and M. R. Green.** 1991. Binding of general transcription factor TFIIB to an acidic activating region. Nature (London) **353:**569–571.
39. **Mitchell, P. J., and R. Tjian.** 1989. Transcriptional regulation in mammalian cells by sequence-specific DNA binding proteins. Science **245:**371–378.
40. **Natesan, S., and M. Z. Gilman.** 1993. DNA bending and orientation-dependent function of YY1 in the c-*fos* promoter. Genes Dev. **7:**2497–2509.
41. **Neuman, E., E. K. Flemington, W. R. Sellers, and W. G. Kaelin, Jr.** 1994. Transcription of the E2F-1 gene is rendered cell cycle dependent by E2F DNA-binding sites within its promoter. Mol. Cell. Biol. **14:**6607–6615.
42. **O'Connor, R. J., and P. Hearing.** 1994. Mutually exclusive interaction of the adenovirus E4-6/7 protein and the retinoblastoma gene product with internal domains of E2F-1 and DP-1. J. Virol. **68:**6848–6862.
43. **Ohtani, K., and J. R. Nevins.** 1994. Functional properties of a *Drosophila* homolog of the E2F1 gene. Mol. Cell. Biol. **14:**1603–1612.
44. **Schreck, R., H. Zorbas, E. Winnacker, and P. A. Baeuerle.** 1990. The NF-κB transcription factor induces DNA bending which is modulated by its 65-kD subunit. Nucleic Acids Res. **18:**6497–6502.
45. **Shan, B., X. Zhu, P. L. Chen, T. Durfee, Y. Yang, D. Sharp, and W. H. Lee.** 1992. Molecular cloning of cellular genes encoding retinoblastoma-associated proteins: identification of a gene with properties of the transcription factor E2F. Mol. Cell. Biol. **12:**5620–5631.
46. **Smith, D. B., and K. S. Johnson.** 1988. Single-step purification of polypeptides expressed in *Escherichia coli* as fusions with glutathione S-transferase. Gene **67:**31–40.
47. **Stringer, K. F., C. J. Ingles, and J. Greenblatt.** 1990. Direct and selective binding of an acidic transcriptional activation domain to the TATA-box factor TFIID. Nature (London) **345:**783–786.
48. **Thompson, J. F., and A. Landy.** 1988. Empirical estimation of protein-induced DNA bending angles: application to lambda site-specific recombination complexes. Nucleic Acids Res. **16:**9687–9705.
49. **Travis, A., A. Amsterdam, C. Belanger, and R. Grosschedl.** 1991. LEF-1, a gene encoding a lymphoid-specific protein with an HMG domain, regulates T-cell receptor alpha enhancer function. Genes Dev. **5:**880–894.
50. **van de Wetering, M., and H. Clevers.** 1992. Sequence-specific interaction of the HMG box proteins TCF-1 and SRY occurs within the minor groove of a Watson-Crick double helix. EMBO J. **11:**3039–3044.
51. **Verrijzer, C. P., J. A. W. M. van Oosterhout, W. W. van Weperen, and P. C. van der Vliet.** 1991. POU proteins bend DNA via the POU-specific domain. EMBO J. **10:**3007–3014.
52. **Waterman, M. L., W. H. Fischer, and K. A. Jones.** 1991. A thymus-specific member of the HMG protein family regulates the human T cell receptor alpha enhancer. Genes Dev. **5:**656–669.
53. **Weintraub, S. J., C. A. Prater, and D. C. Dean.** 1992. Retinoblastoma protein switches the E2F site from positive to negative element. Nature (London) **358:**259–261.
54. **Yee, A. S., P. Raychaudhuri, L. Jakoi, and J. R. Nevins.** 1989. The adenovirus-inducible factor E2F stimulates transcription after specific DNA binding. Mol. Cell. Biol. **9:**578–585.
55. **Zinkel, S. S., and D. M. Crothers.** 1987. DNA bend direction by phase sensitive detection. Nature (London) **328:**178–181.