cAMP-response Element-binding Protein Induces Directed DNA Bending of the HTLV-I 21-Base Pair Repeat*

(Received for publication, September 7, 1995, and in revised form, December 12, 1995)

Min J ean Yint, Eysvind Paulsens, and Richard B. Gaynor§

From the Divisions of Molecular Virology and Hematology-Oncology, Departments of Internal Medicine and Microbiology, University of Texas Southwestern Medical Center, Dallas, Texas 75235-8594

Gene expression from the human T-cell leukemia virus type I (HTLV-I) long terminal repeat (LTR) is mediated by three cis-acting regulatory elements known as 21-base pair (bp) repeats in addition to the transactivator protein Tax. Each of the 21-bp repeats contain nucleotide sequences which are homologous to a cAMP response element (CRE) which bind members of the ATF/CREB family of transcription factors. In this study, we investigated whether CREB alone or in the presence of Tax was able to induce DNA structural changes when bound to CRE sites in the HTLV-I 21 bp, the cellular somatostatin promoter, or a hybrid CRE construct comprised of both the somatostatin and 21-bp repeat sequences. Circular permutation analysis indicated that CREB was able to induce DNA flexure upon binding to each of these elements. However, phasing analysis, which is a more sensitive method to determine the degree and orientation of directed DNA bending, demonstrated that CREB induced DNA bending of the HTLV-I 21-bp repeat and the hybrid CRE but not the somatostatin CRE. The addition of Tax did not change CREB-mediated bending of the 21-bp repeat or the hybrid CRE although it markedly increased the amount of CREB bound to each of these DNA elements. These results indicate that sequence motifs flanking the CRE in the 21-bp repeat are critical for inducing DNA structural changes and that these changes are likely important in mediating Tax activation of the HTLV-I LTR.

The HTLV-I long terminal repeat (LTR) contains three cis-acting regulatory elements designated 21-bp repeats which are necessary for transactivation by Tax (16, 17). The sequences within the 21-bp repeats have been further subdivided into three motifs designated A, B, and C (18). The A and C motifs which are GC-rich sequences flanking the B motif are involved in controlling the level of Tax activation. The B motif in each of the 21-bp repeats is designated as the tax-response element and is highly homologous to a cyclic AMP-response element (CRE) which is found in a variety of genes whose expression is increased in response to elevated levels of cAMP by binding members of the ATF/CREB family of transcription factors (19). The B motif mediates increases in HTLV-I gene expression in response to Tax and serves as the binding site for a variety of members of the ATF/CREB family (20–22). In contrast to the HTLV-I LTR, Tax activation of viral gene promoters is frequently mediated by cellular transcription factors other than ATF/CREB. For example, Tax activation of interleukin-2 receptor and the HIV-1 LTR is mediated by increases in the level of NF-κB (13, 15, 23, 24) while Tax activation of the c-fos and the parathyroid hormone-related protein genes are controlled by modulation of the cellular factors SRF and AP-2, respectively (14, 25). Thus, it is likely that Tax may either directly or indirectly interact with a variety of different transcription factors to increase the gene expression from Tax-responsive genes. Tax stimulates the binding of a number of members of ATF/CREB family to the 21-bp repeats (22, 26–32). The mechanism of this stimulation remains unclear since several studies do not demonstrate stable binding of Tax to gel-retarded complexes containing CREB (26–28, 31). This may be due to dissociation of Tax during gel electrophoresis. Using both in vitro binding studies and the mammalian two-hybrid system, we find that Tax specifically interacts with CREB but not other members of the ATF/CREB family or other leucine zipper proteins (26). CREB interaction with Tax is mediated by its basic leucine zipper domain which is also required for its dimerization and DNA binding properties (33, 34). A variety of such bZIP proteins including Fos-Jun, in addition to other members of the ATF/CREB family, bind to their cognate DNA recognition elements by forming dimeric complexes (35–37). Tax stimulation of CREB binding to the 21-bp repeat may result from its ability to either enhance dimerization of CREB, change the conformation of CREB, or alter the DNA structure of the 21-bp repeat (31, 38).

DNA is frequently distorted from its regular double helical structure by the binding of cellular transcription factors. A well described and experimentally accessible mode of DNA distortion is DNA bending (35–37, 39–41). Induction of DNA bending has been observed for several prokaryotic proteins such as the Escherichia coli CAP and IHF proteins (40, 42–44), the λ O protein (45) and a variety of eucaryotic transcriptional regula-

---

*This work was supported by grants from the Council of Tobacco Research. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

† Both authors contributed equally to this work.

§ To whom correspondence should be addressed: Div. of Molecular Virology, Dept. of Internal Medicine, University of Texas Southwestern Medical Center, 5323 Harry Hines Blvd., Dallas, TX 75235-8594. Tel.: 214-648-7570; Fax: 214-648-8862.

1 The abbreviations used are: HTLV-I, human T-cell leukemia virus type I; HIV-I, human immunodeficiency virus, type I; LTR, long terminal repeat; CRE, cAMP response element; CREB, cAMP response element-binding protein; bp, base pair(s).
tors including the Drosophila heat-shock transcription factor (26), Xenopus laevis heat-shock transcription factor (46), and the Jun-Fos containing AP-1 complex (35). Furthermore, induction of DNA bending by these transcription factors is not a mere side effect of DNA-protein complex formation, but it is of functional significance in many instances (47, 48). Since several bZIP proteins can induce distinct changes in DNA structure upon stable binding to their cognate binding sites (35, 36), we wished to determine whether CREB could induce DNA bending of either the HTLV-I 21-bp repeat or the somatostatin CRE. Furthermore, we wanted to determine whether Tax was able to facilitate CREB-mediated DNA bending using both circular permutation and phasing analysis. Our results using phasing analysis indicate that CREB can be able to induce directed DNA bending of both the HTLV-I 21-bp repeat and a hybrid construct containing the somatostatin CRE flanked by the 21-bp repeat A and C motifs. However, CREB does not induce DNA bending of the somatostatin CRE. Although Tax markedly stimulates both the amount and stability of CREB bound to the HTLV-I 21-bp and the hybrid CRE, it does not itself modify CREB-induced DNA bending. These results suggest that the A and C motifs flanking the CRE are critical for CREB-induced structural changes in the 21-bp repeat. Such structural changes in the 21-bp repeats are likely important for CREB-mediated induction of HTLV-I gene expression.

EXPERIMENTAL PROCEDURES

Plasmid Constructs—Oligonucleotides containing the HTLV-I LTR 21-bp repeat (26) were cloned into the SalI site of the circular permutation vector pBend (21) to generate the derivative plasmids pBend-21 bp repeat, pBend-CRE, and pBend-hybrid CRE, respectively. The sequences of the sense strand of each of the oligonucleotides are: 5′-TCGACAGGCGTTGACGACAACCCCT-3′; somatostatin CRE, 5′-TCGACACCTTGGCTAGTCAGGACAGAGACAA-3′; and the hybrid, 5′-TTCGACACCTTGGCTAGTCAGGACAGAGACAA-3′. Constructs for phasing analysis were generated by the insertion of a set of five double-stranded oligonucleotides containing three adenine tracts (5′-TCGACCTTTTICTGGCCCTTTGCTTTTGC-3′) and different linker elements containing either AG for 2 bp, CGAG for 4 bp, CAGCAG for 6 bp, and CAGCCTGACG for 8 bp inserted into the SalI-digested pBend plasmid (21). The resultant plasmids were designated pPhaseI(2), pPhaseI(4), pPhaseI(6), pPhaseI(8), and pPhaseI(10), respectively. The plasmids used for phasing analysis consisted of the oligomers used in the permutation clones inserted into the five pPha-seII vectors (39), resulting in a distance between the middle of the A-tract and the middle of the CRE binding site of 25, 27, 31, and 33 base pairs, respectively. Oligonucleotides containing the HTLV-I 21-bp III, somatostatin CRE, and the hybrid 21-bp repeat-CRE were cloned into each of these pPhaseI plasmids (41).

To construct 5′ nucleotide deletions between each of the 21-bp repeats or the 21-bp repeats and the TATA box in vitro mutagenesis of the HTLV-I LTR in M13 was performed using the Sculptor site-directed mutagenesis kit (Amersham) using the oligonucleotides: CTCCCCCAGGAGGACACCGGCCCTACAGCT-GGCC (Δ5 bp I), GTTCCTCAGAGACAGACACTATCCCCTCGGAG (Δ5 bp II), GTATTCCCTCAGAGC-3′; somatostatin CRE, CTGCAGATGCTTGGCTAGTCAGGACAGAGACAA-3′; and the hybrid, 5′-TTCGACACCTTGGCTAGTCAGGACAGAGACAA-3′. The resulting products from all mutagenesis reactions were purified by agarose gel electrophoresis (2% agarose in TAE) and cloned into the EcoRI site of pBluescript KS1(−) containing the TATA box, APH is the amplitude of phasing function, aI is the intrinsic DNA bend angle, αo is the protein-induced bend angle, and αo plus αI is less than 120°. Determination of the absolute orientation is derived from Ref. 37, 38.

\[ \frac{\beta_0}{\beta} = 360 \times \frac{2\pi}{\beta} \text{ modulo } 360 \]  

(6)

where \( \beta_0 \) is the DNA bend orientation relative to the major groove minor axis, \( \beta \) is the transverse length, \( \beta_0 \) is phasing period. Transfections and Chimeric polynucleotide transfection assays—Jurkat cells were maintained in RPMI containing 10% fetal bovine serum with penicillin and streptomycin. DEAE transfections were performed.
Tax stimulation of CREB binding is dependent on sequences flanking the CRE. A, either 10 ng (lanes 2, 3, 7, 8, 12, and 13) or 30 ng (lanes 4, 5, 9, 10, 14, and 15) of CREB protein was incubated with 50,000 cpm of probe corresponding to the HTLV-I 21-bp repeat (lanes 1-5), the somatostatin CRE site (lanes 6-10), or the hybrid CRE (lanes 11-15) in the presence (lanes 3, 5, 8, 10, 13, and 15) or absence (lanes 2, 4, 7, 9, 12, and 14) of Tax, and the DNA-protein complexes were analyzed by electrophoretic mobility shift assay (EMSA). Lanes 1, 6, and 11 contain labeled probe in the absence of CREB. B, gel retardation was performed with the 21-bp repeat probe (lane 1) and incubated with either CREB (30 ng) alone (lane 2) or with the addition of 50 ng of either wild type Tax (lane 3) or an N-terminal truncation of Tax (lane 4). C, gel retardation assays were performed with an increasing amount of CREB ranging from 5 to 120 ng in both the presence and absence of 50 ng of Tax. The radioactivity of the DNA-protein complexes were quantitated by PhosphorImager scanning and the amount that Tax stimulated CREB binding to each set of oligonucleotides was plotted as a function of increasing quantities of CREB.

by adding 5 μg of either the wild-type or mutant HTLV-I constructs in both the presence and absence of 1 μg of a wild-type or mutated Tax expression vector and a β-galactosidase control plasmid (50). Cells were harvested after 48 h and one-fourth of each of the cell extract, normalized for β-galactosidase activity, was used for chloramphenicol acetyltransferase assays. All transfections were repeated three times.

RESULTS

Tax Stimulates CREB Binding to the HTLV-I 21-bp Repeat but Not to the Somatostatin CRE—Each of the HTLV-I 21-bp repeats contain non-palindromic CRE sites in contrast to the palindromic sites found in the somatostatin promoter (17, 49, 51). Before analyzing the role of CREB and Tax on inducing DNA structural changes of the 21-bp repeat and the somatostatin CRE, gel retardation analysis was performed with each of these elements to compare the binding of the bacterial expressed CREB in both the presence and absence of Tax. CREB bound weakly to oligonucleotides corresponding to 21 bp repeat III (Fig. 1A, lanes 2 and 4). In contrast, a much greater degree of CREB binding to somatostatin CRE was observed (Fig. 1A, lanes 7 and 9). Tax was able to markedly stimulate CREB binding to HTLV-I 21-bp repeat III using both low and high concentrations of CREB (Fig. 1A, lanes 3 and 5). Similar results were also noted using 21-bp repeats I and II (data not shown). In contrast, there was little (Fig. 1A, lanes 7 and 8) or no (Fig. 1A, lanes 9 and 10) Tax-induced stimulation of CREB binding to the somatostatin CRE. Since the A and C motifs of the 21-bp motif are critical for Tax activation, hybrid oligonucleotides which contained these motifs flanking the palindromic CRE or the wild-type CRE promoter were tested for their ability to bind CREB in the presence and absence of Tax. Tax was able to stimulate the binding of CREB to the hybrid CRE (Fig. 1A, lanes 12-15) indicating the critical nature of sequences flanking the CRE for Tax-mediated stimulation of CREB binding. An amino-terminal truncation of Tax which did not interact with CREB using the mammalian two-hybrid system (26) was unable to stimulate CREB binding to the 21-bp repeat indicating the specificity of Tax stimulation (Fig. 1B, lanes 2-4). Although Tax markedly stimulated CREB binding, no slower mobility complex potentially comprised of both the Tax and CREB proteins was detected.

To better quantitate the differences in Tax stimulation of CREB binding using these probes, we performed gel retardation assays with Tax in the presence of increasing concentrations of CREB. CREB binding was quantitated by PhosphorImager analysis and the amount of Tax stimulation was plotted as a function of the concentration of CREB (Fig. 1C). These results indicated that Tax increased CREB binding to the HTLV-I 21-bp repeat approximately 4-fold using low concentrations of CREB but only 2-fold when higher amounts of CREB were used (Fig. 1C). A similar degree of Tax stimulation of CREB binding was also noted using the hybrid CRE (Fig. 1C). In contrast, Tax was unable to increase CREB binding to somatostatin CRE site at either low or high concentrations of CREB (Fig. 1C). These results indicated that the DNA sequences flanking the CRE were critical for regulating both the amount of CREB binding and the degree of Tax-stimulation.

CREB Induces DNA Structural Changes in the HTLV-I 21-bp Repeat, Hybrid CRE, and the Somatostatin CRE—Since an increasing number of both procaryotic and eucaryotic transcription factors have been demonstrated to mediate interactions by bending DNA upon binding to their recognition sites, we wanted to determine whether CREB alone or in the presence of Tax resulted in DNA structural changes. Bending of DNA upon the binding of transcription factors can be detected by the anomalous migration of protein-DNA complexes in polyacrylamide gels (40). Thus, anomalous migration is maximized when the DNA-protein binding site is located in the center of a fragment and minimized when it is located near the end of the fragment (35, 40, 42–47). Oligonucleotides containing either the HTLV-I 21-bp repeat, the somatostatin CRE, or the hybrid CRE were cloned into the pBend2 vector (39) and used to perform circular permutation analysis (43). Digestion with a number of different restriction endonucleases generated fragments of identical size (145 bp), which varied in the position of the DNA binding site (Fig. 2).

First, we analyzed the electrophoretic mobility of circularly
permutated fragments containing the HTLV-I 21-bp repeat which were incubated with CREB in either the presence or absence of Tax (Fig. 3). The bound and free probes are shown in separate panels due to the prolonged times required for gel electrophoresis because of the extremely slow mobility of the CREB-DNA complexes. No obvious anomalous migration was observed for the free DNA probes indicating that they did not have any intrinsic curvature (Fig. 3B, lanes 1-10). Migration of the complex containing the 21-bp repeat and CREB was clearly dependent on the position of the binding site within the different pBend fragments which was suggestive of CREB-induced DNA structural changes (Fig. 3A, lanes 1-10). This was reflected by the fact that CREB-DNA complexes migrated slower when bound to probes in which the 21-bp repeat was in the middle of the fragment as compared to fragments where the binding sites were located at either end of the fragment. The relative migration of the CREB-DNA complexes did not significantly change in either the presence or absence of Tax although there was a significant increase in CREB binding in the presence of Tax (Fig. 3A, lanes 1-10). This is reflected in the graph shown in Fig. 3C which indicates a similar pattern of mobility for each of the CREB-21-bp repeat complexes in the presence and absence of Tax. It should be noted that the increased amount of CREB binding to the 21-bp repeat in the presence of Tax would also be consistent with the presence of Tax in the gel retarded complex with CREB. However, addition of Tax antibody to these gel retardation assays did not result in a supershift of the gel retarded complex (data not shown).

The electrophoretic mobility of circularly permuted fragments containing the somatostatin CRE was next performed with CREB in both the presence and absence of Tax (Fig. 3A, lanes 1 and 10). CREB induced DNA structural distortion when bound to the somatostatin CRE site (Fig. 3A, lanes 1 and 10). The presence of Tax did not change the relative mobility of the somatostatin CRE in the presence of CREB nor did it induce CREB binding (Fig. 4, A and C). Finally, a similar analysis was performed using the hybrid oligonucleotide which contained the somatostatin CRE flanked by the 21-bp repeat A and C motifs (Fig. 5). The changes in the mobility of the fragments containing the hybrid CRE in the presence of CREB were similar to the results obtained with the somatostatin CRE and the 21-bp repeat (Fig. 5, A and C). There were no changes in the mobility of the hybrid CRE bound to CREB in either the presence or absence of Tax, although Tax markedly stimulated CREB binding (Fig. 5, A and C). A variety of previous studies have demonstrated that a variety of other bZIP proteins display position-dependent variations in electrophoretic mobility upon binding to their cognate binding sites. Our results indicate that CREB induces similar DNA structural distortions in the HTLV-I 21-bp repeat, the somatostatin CRE site, and the hybrid CRE with flexure angles ranging from 40° to 49° and that these CREB-induced DNA structural changes were not influenced by the presence of Tax (Table I). The flexure angles seen upon the binding of other bZIP proteins to their cognate sites vary with the Fos-J un heterodimer having a flexure angle of 94° and the Jun homodimer having a flexure angle of 79° (36, 37).

Phasing Analysis Demonstrates that CREB induces DNA Bending of the 21-bp Repeat and the Hybrid CRE but Not the Somatostatin CRE—Circular permutation analysis does not allow a determination of specific DNA bending, because it also detects distortion in DNA structure such as regions of increased DNA flexibility. Phasing analysis introduces an approach for determining the relative DNA bending angle and the direction of the DNA bend by using isomers containing helical

[Figure 2: Structure of the restriction fragments used in circular permutation analysis. Oligonucleotides containing the HTLV-I 21-bp repeat III, the somatostatin CRE, and the hybrid CRE were cloned into the pBend vector and then digested with MluI (A), SpeI (B), PvuII (C), NruI (D), and BamHI (E) to generate the fragments used in circular permutation analysis. The position of the CRE in each of the oligonucleotides is indicated by shading.

[Figure 3: Circular permutation assay of CREB binding to the HTLV-I 21-bp repeat. A, the labeled pBend DNA fragments (A-E) containing the 21-bp repeat oligonucleotides were incubated with 50 ng of CREB in the presence (lanes 2, 4, 6, 8, and 10) or absence of (lanes 1, 3, 5, 7, and 9) Tax and the DNA-protein complexes analyzed by electrophoretic mobility shift assay are shown. B, the mobilities of the free probes (A-E) are shown. C, the ratio of the mobilities of the HTLV-I 21-bp CREB complex relative to that of the corresponding free DNA was plotted as a function of the position of the binding site.

[Figure 4: Phasing Analysis Demonstrates that CREB Induces DNA Bending of the 21-bp Repeat and the Hybrid CRE but Not the Somatostatin CRE. The changes in the mobility of the fragments containing the hybrid CRE in the presence of CREB were similar to the results obtained with the somatostatin CRE and the 21-bp repeat (Fig. 5, A and C). There were no changes in the mobility of the hybrid CRE bound to CREB in either the presence or absence of Tax, although Tax markedly stimulated CREB binding (Fig. 5, A and C). A variety of previous studies have demonstrated that a variety of other bZIP proteins display position-dependent variations in electrophoretic mobility upon binding to their cognate binding sites. Our results indicate that CREB induces similar DNA structural distortions in the HTLV-I 21-bp repeat, the somatostatin CRE site, and the hybrid CRE with flexure angles ranging from 40° to 49° and that these CREB-induced DNA structural changes were not influenced by the presence of Tax (Table I). The flexure angles seen upon the binding of other bZIP proteins to their cognate sites vary with the Fos-J un heterodimer having a flexure angle of 94° and the Jun homodimer having a flexure angle of 79° (36, 37).]
phasing between two DNA bends (41). It provides a more specific method for the analysis of DNA bending allowing discrimination between directed DNA bends and regions of increased DNA flexibility (52). In addition, the orientation of the bend relative to the reference standard in the fragment can be determined.

We cloned oligonucleotides corresponding to the HTLV-I 21-bp repeat, the somatostatin CRE, and the hybrid CRE into the pPhaseII vector (41) which contains three adenine tracts that intrinsically bend DNA (Fig. 6). Using a set of linkers varying by 2 bp in length from 2 to 10 nucleotides, the distance between the CREB binding site and the adenine tract-directed bend was altered (41). Thus, the length of the spacer was varied over one turn of the DNA helix to place the CREB binding site on different faces of the DNA relative to the DNA bend. If CREB binding induced DNA bending, the mobility of the CREB-DNA complex should depend on the linker length since this determines the relative orientation of the protein-induced DNA bend. The mobility of the DNA-protein complex was minimal in the cis-isomer where the end-to-end distance is short and maximal in the trans-isomer.

Gel retardation assays were performed with CREB and the 21-bp repeat (Fig. 7, A and B) in addition to the position of the free probes (Fig. 7C) are shown. Electrophoresis of these gel retardation assays was performed for both 4 (Fig. 7A) and 12 (Fig. 7B) h to accentuate potential CREB induced effects on DNA bending. These prolonged times of electrophoresis resulted in marked dissociation of CREB from the 21-bp repeat in the absence but not the presence of Tax (Fig. 7B). This effect was not seen during shorter periods of electrophoresis (Fig. 7A). The relative mobilities of the CREB-21 bp repeat complexes in both the presence and absence of Tax were obtained upon long exposure of the autoradiogram in Fig. 7C and divided by the relative mobilities of free DNA. No 10-bp periodicity was noted using the five phasing probes containing the 21-bp repeat (Fig. 7C). A clear variation with a 10-bp periodicity was observed when these relative mobilities were plotted against the linker length (Fig. 7D). Although Tax markedly altered the stability of the CREB-21 bp repeat complex following prolonged gel electrophoresis, it did not alter the 10-bp periodicity observed upon the binding of CREB alone to the 21-bp repeat. This finding was confirmed in three independent experiments (Fig. 7D).

It was important to calculate the orientation and degree of CREB-induced bending. Phased A:T tracts bend DNA toward the minor groove at the center of the A:T tract (41). Proteins
Circular permutation analysis from three different experiments was used to determine the mobility anomaly, circular amplitude, and flexure angle for the somatostatin CRE, HTLV-I 21-bp repeat, and the hybrid CRE in both the presence and absence of Tax. Phasing analysis from three different experiments was used to determine the phasing amplitude, directed bend angle, and the bend orientation for the three binding sites. Equations used to calculate these functions are described under "Experimental Procedures."

| DNA fragment | Mobility anomaly (\(\mu_{\text{mob}}\)) | Circular amplitude (\(A_{\text{CP}}\)) | Flexure angle (\(\alpha_p\)) | Phasing amplitude (\(A_{\text{ph}}\)) | Directed bend angle (\(\alpha_p\)) | Bend orientation (\(\beta_p\)) |
|--------------|----------------------------------|---------------------------------|--------------------------|---------------------------------|------------------------------|---------------------------|
| (-) Tax      | Somat CRE                        | 0.937 ± 0.013                   | 0.071 ± 0.001            | 40.89 ± 1.59                    | 0.752 ± 0.021                | ND*                       | ND                        |
|              | 21-bp repeat                     | 0.913 ± 0.015                   | 0.098 ± 0.001            | 48.14 ± 1.23                    | 0.546 ± 0.011                | 9.27 ± 0.98               | 144                       |
|              | Hybrid CRE                       | 0.913 ± 0.016                   | 0.096 ± 0.001            | 47.93 ± 1.21                    | 0.662 ± 0.020                | 8.52 ± 0.83               | 144                       |
| (+) Tax      | Somat CRE                        | 0.936 ± 0.015                   | 0.071 ± 0.001            | 41.05 ± 1.62                    | 0.771 ± 0.019                | ND*                       | ND                        |
|              | 21-bp repeat                     | 0.913 ± 0.016                   | 0.104 ± 0.001            | 49.62 ± 1.31                    | 0.508 ± 0.024                | 9.24 ± 1.05               | 144                       |
|              | Hybrid CRE                       | 0.922 ± 0.014                   | 0.087 ± 0.001            | 45.64 ± 1.44                    | 0.638 ± 0.026                | 8.01 ± 0.79               | 144                       |

*ND, not detectable.

Results seen with the 21-bp repeat, prolonged electrophoresis did not markedly alter the amount of CREB bound to the CRE in either the presence or absence of Tax (Fig. 8, A and B). The phasing probes containing the somatostatin CRE are shown in Fig. 8C. In contrast to the results seen with the 21-bp repeat probe, there was a 10-bp periodicity seen using the five phasing probes in the somatostatin CRE (Fig. 8C). However, unlike the results with the 21-bp repeat, there was no 10-bp periodicity observed when the relative mobilities of the CREB-somatostatin CRE complexes were divided by the relative mobilities of free DNA and plotted against the linker length (Fig. 8D). Although CREB binding to the somatostatin CRE was associated with changes in DNA flexibility using circular permutation analysis, there was no directed DNA bending found using the more specific phasing analysis as previously determined (37).

Since CREB was able to induce phasing with the 21-bp repeat but not the somatostatin CRE, we next determined whether this effect was dependent primarily on the sequences of the CRE or the flanking A and C motifs. Phasing analysis was performed using CREB and the hybrid CRE construct in the presence and absence of Tax (Fig. 9). With prolonged periods of electrophoresis, CREB dissociated from the hybrid CRE in the absence but not the presence of Tax (Fig. 9B) which did not occur during shorter periods of electrophoresis (Fig. 9A). This suggested that the A and C motifs were critical for Tax-mediated stabilization of CREB binding. The mobility of the phasing probes containing the hybrid CRE indicated that there was no 10-bp periodicity (Fig. 9C).

It was thus critical to determine whether the A and C motifs influenced the degree of CREB-induced bending. When the relative mobilities of complexes composed of CREB bound to the hybrid CRE were divided by the relative mobilities of free DNA and plotted against the linker length, a clear variation with a 10-bp periodicity was observed (Fig. 9D). The presence of Tax did not alter the 10-bp periodicity observed with CREB alone (Fig. 9, A and B). Thus, phasing analysis with the hybrid CRE gave similar results to that seen with the 21-bp repeat indicating that a 9° bend toward the minor groove occurred upon CREB binding to the hybrid CRE. These results suggest that the GC-rich sequences present in the A and C motifs flanking the CRE are required for both CREB-induced DNA bending and Tax stimulation of CREB binding. Whether structural changes in the 21-bp repeat are a requirement for subsequent Tax stimulation of gene expression remains to be determined.

Alterations in spacing of the HTLV-I 21-bp repeats do not alter basal and Tax-induced gene expression—Examination of the HTLV-I LTR indicates that there is an exact 80-bp spacing...
between the B motifs in the three 21-bp repeats thus placing
the three 21-bp repeats on the same side of the DNA helix (17,
51). The transcription factor YY1 binds to three sites in the
c-fos promoter and induces a phased DNA bend that facilitates
interactions between CRE and TATA binding sites (53). Inser-
tion of 5 bp of DNA between either the YY1 or CREB binding
sites and the TATA element in the c-fos promoter place the YY1
and CREB sites on opposite sides of the DNA relative to the
TATA element (53). This insertion resulted in changes in tran-
scription indicating that YY1 functions to maintain optimal
spacing between the CRE and the TATA element (53). Similar
dependence on DNA structure has been noted with other tran-
scription factors such as LEF-1 (54) and HMG I(Y) (55).

**FIG. 7.** Analysis of directed DNA bending of the HTLV-I 21-bp
repeat by phasing analysis. Phasing analysis was performed with
probes containing the 21-bp sequences flanked by different linker
lengths and incubated with 50 ng of CREB in either the presence (lanes
2, 4, 6, 8, and 10) or absence (lanes 1, 3, 5, 7, and 9) of Tax followed by
gel electrophoresis. The position of the bound probe following gel elec-
trophoresis for either (A) 4 or (B) 12 h is shown. C, the position of the
free probe is also shown and the linker length is indicated on top of each
lane. D, the ratio of the relative mobilities of the CREB-DNA complex
divided by the relative mobilities of the corresponding free DNA was
plotted against linker length in three independent experiments and the
standard deviation included.

**FIG. 8.** Analysis of directed DNA bending of the somatostatin
CRE by phasing analysis. Phasing analysis was performed with
probes containing the somatostatin CRE sequences flanked by different
linker lengths and incubated with 50 ng of CREB either in the presence
(lanes 2, 4, 6, 8, and 10) or absence (lanes 1, 3, 5, 7, and 9) of Tax
followed by gel electrophoresis. The position of the bound probe following
gel electrophoresis for either (A) 4 or (B) 12 h is shown. C, the
position of the free probe is also shown and the linker length is indi-
cated on top of each lane. D, the ratio of the relative mobilities of the
CREB-DNA complex divided by the mobilities of the corresponding free
DNA was plotted against linker length in three independent experi-
ments and the standard deviation included.
following transfection of Jurkat cells. There were no significant differences in the level of gene expression with these constructs as compared to the wild-type HTLV-I construct (Fig. 10). These results suggest that even though CREB is able to induce bending of the 21-bp DNA, DNA bending does not result in overall changes in the structural integrity of the HTLV-I LTR which alter interaction between the different 21-bp repeats.

However, the sequences flanking the CRE in the 21-bp repeat are critical for Tax activation. Wild-type and mutant HTLV-I constructs and the somatostatin CRE were assayed following transfection of these constructs in either the presence or absence of Tax. The constructs that were assayed included the wild-type HTLV-I LTR, HTLV-I LTR mutants in which the A (NBC), C (ABN), or A and C (NBN) sequences flanking the 21-bp repeat CRE were mutated, an HTLV-I hybrid in which the CRE in each of the 21-bp repeats was changed to the somatostatin CRE sequences but the 21-bp repeat flanking sequences were maintained, and the somatostatin CRE (Table II). These results demonstrated that Tax increased HTLV-I gene expression 6.5-fold, while mutations of either one (NBC and ABN) or both (NBN) of the flanking sequences in the 21-bp repeats resulted in nearly a complete loss of Tax activation (Table II). Mutations which change each of the CRE sites in the HTLV-I 21-bp repeats to that of the somatostatin CRE resulted in approximately a 3-fold increase in Tax activation as compared to the wild-type HTLV-I LTR (Table II). Finally, gene expression of the somatostatin promoter was not activated by Tax (Table II). These results indicate that Tax activation is dependent on the sequences flanking the 21-bp repeat CRE and demonstrate that in vivo Tax activation correlates with the ability of CREB to bend DNA in phasing analysis.

**DISCUSSION**

Each of the three 21-bp repeats in the HTLV-I LTR contain a non-palindromic CRE (16, 17, 51) which serve as the binding sites for members of the ATF/CREB family. Tax stimulates CREB binding to each of these 21-bp repeats and this effect is likely critical for subsequent Tax activation of gene expression. However, Tax only weakly stimulates CREB binding to the palindromic CRE found in the somatostatin promoter indicating that differences in DNA sequences between the 21-bp repeat and the somatostatin CRE are critical for Tax stimulation. Previous studies indicate that a variety of bZIP proteins such as Fos and Jun are able to induce a variety of topologically distinct DNA structures at AP-1 binding sites (35–37). For example, Fos-Jun heterodimers and Jun homodimers induce DNA bends that are directed in opposite orientations (36) whereas CREB and ATF1 do not induce significant bending when bound to the CRE site (36, 37). Since CREB is a classical bZIP protein (33, 34) and stimulation of its binding to the HTLV-I 21-bp repeat by Tax is highly sequence dependent, it was important to delineate whether CREB induces DNA structural changes in the HTLV-I 21-bp repeat as compared to the somatostatin CRE. The role of the CRE versus flanking DNA sequences was also addressed. Furthermore, we wished to determine whether DNA bending was associated with the ability of Tax to stimulate CREB binding to DNA.

Circular permutation analysis indicates that CREB induces DNA structural distortion in the HTLV-I 21-bp repeat, the somatostatin CRE, and a hybrid CRE comprised of the somatostatin CRE flanked by the A and C motifs from the 21-bp repeats. However, Tax does not change the pattern of CREB-induced DNA structural distortion although it is able to markedly stimulate CREB binding to the 21-bp repeat and hybrid CRE. A variety of previous studies indicate that circular permutation analysis will detect changes in DNA flexibility rather than directed DNA binding which is detected by phasing analysis. For example, several members of the ATF/CREB family
have been shown to induce marked changes of CRE DNA flexure in circular permutation analysis although they induce no changes in phasing analysis (37). Likewise the helix-loop-helix proteins TFEB, TFE3, USF, Myc, and Max induce significant DNA flexure using circular permutation analysis (56, 57). Phasing analysis demonstrates that TFEB, TFE3, USF, Max, and Myc-Max dimers bend DNA in the same orientation (56) and Myc homodimers bend DNA in the opposite orientation (57) although these proteins induce relatively small DNA bending angles.

Phasing analysis was performed to determine the degree of CREB-induced DNA bending and the orientation of this bending. The results using phasing analysis indicate that CREB induces modest but reproducible directed DNA bending of the 21-bp repeat and the hybrid CRE, but does not induce bending of the somatostatin CRE. These results support previous observations that circular permutation analysis is not a reliable method for the determination of directed DNA bends and that although ATF/CREB proteins induce DNA mobility variation in circular permutation analysis they are unable to induce directed DNA bending in CRE site or in AP-1 sites (37, 38). The inability of CREB to induce bending of the CRE has been suggested to result from intrinsic bending of the CRE sequence into a conformation that enhances CREB binding (38). Our results are in agreement with this previous study and indicate that the somatostatin CRE is pre-bent but the binding of CREB eliminates this bending. Circular dichroism spectroscopy demonstrates that the DNA-binding domains of several bZIP proteins undergo conformational transitions to structures of a high helix content in the presence of their cognate DNA-binding site (58–60). Since CREB binding induces different DNA structural changes in the HTLV-I 21-bp repeat and somatostatin CRE, it is possible that the CREB protein adopts different conformations upon binding to different DNA binding sites. According to thermodynamic principles, a conformational change in the 21-bp repeat and the hybrid CRE, which is dependent on the sequences found in the A and C motifs, could generate an energy barrier preventing efficient CREB binding. This may explain why CREB binding to the pre-bent CRE site occurs with higher affinity than to the unbent CRE sequences found in the 21-bp repeat. A similar mechanism occurs with TBP which binds to pre-bent DNA with 100-fold higher affinity than unbent DNA of identical sequence (61). Although Tax does not alter the CREB-induced DNA bending angle, it may alter the CREB conformation to overcome a high energy barrier to CREB binding to the 21-bp repeat.

One of the intriguing features of this study is that prolonged electrophoresis of the 21-bp repeat and the hybrid CRE during gel electrophoresis results in dissociation of CREB in the absence but not the presence of Tax. However, no similar effect is seen with prolonged electrophoresis of CREB binding to the somatostatin CRE. These results suggest that Tax increases the stability of CREB binding to the 21-bp repeat and the hybrid CRE. Whether this effect is mediated by the presence of Tax in the DNA complex with CREB or whether Tax induces a conformational change in CREB remains to be determined. The inability in many studies to supershift Tax in the complex with CREB would favor the latter possibility (26–28, 31). The failure of Tax to change the CREB-induced binding angle of the 21-bp repeat and hybrid CRE would also be consistent with the presence of CREB alone in the complex bound to DNA. However, these possibilities need to be addressed by performing structural studies of the CREB-Tax complex bound to different CRE sites. Our results support previous data that subtle differences in the DNA structure of CRE and AP-1 sites result in marked differences on the binding and subsequent bending by the bZIP family of proteins (37, 38).

DNA bending may promote or inhibit the binding of proteins that recognize overlapping or adjacent DNA elements. Intrinsic DNA bends have been shown to markedly influence DNA bind-
ing by several eucaryotic transcription factors (62). Therefore, CREB binding to the 21-bp repeat and subsequent induction of DNA bending may help to recruit a variety of cellular transcription factors or prevent inhibitors from binding to this important regulatory element. Cellular transcription factors which are recruited to CREB bound to the 21-bp repeat may be important in the regulation of HTLV-I gene expression. For example, it will be critical to address whether factors such as p300 which are able to associate with phosphorylated form of CREB (63) are able to bind to the 21-bp repeat. However, our results indicate that CREB-induced DNA bending is dependent on the sequences flanking the CRE and that these sequences are also critical for Tax activation. Future studies will be required to understand the role of cellular factors which bind to the HTLV-I LTR and how changes in the DNA structure modulate HTLV-I gene expression.

Acknowledgments—We thank Sharon Johnson for manuscript preparation and Susan Vaughn for preparation of the figures.

REFERENCES
1. Hinuma, Y., Komoda, H., Chosa, T., Kondo, T., Kohakura, M., Takenaka, T., Kikuchi, M., Ichimaru, M., Yunoki, K., Satô, I., Matsuo, R., Takuchi, Y., Udono, H., and Hanaoka, M. (1982) Int. J. Cancer 29, 631–635
2. Polesz, B. J., Rusotti, R. W., Gazdar, A. F., Bunn, P. A., Minna, J. D., and Gallo, R. C. (1983) Proc. Natl. Acad. Sci. U. S. A. 77, 7415–7419
3. Gessain, A., Barin, F., Vernant, J. C., Gout, O., Maurs, L., Calender, A., and De Decker, B. (1985) Nature 318, 229, 54–58
4. Sodroski, J. G., Peifer, M., Faustino, N. A., Brenchley, J. M., and Fauci, A. S. (1993) Proc. Natl. Acad. Sci. U. S. A. 90, 11445–11449
5. Cross, S. L., Feinstein, M. B., Wolf, J. B., Holbrook, N. J., Wong-Staal, F., and Greene, W. C. (1987) Science 236, 1575–1579
6. Montminy, M. R., Sevarino, K. A., Wagner, J. A., Mandel, G., and Goodman, R. H. (1992) Proc. Natl. Acad. Sci. U. S. A. 89, 6899–6903
7. Syder, U. K., Thompson, J. F., and Landy, A. (1989) Nature 341, 252–254
8. Virelli, L., Tottori, T., and Miwa, M. (1986) Proc. Natl. Acad. Sci. U. S. A. 83, 713–718
9. Natesan, S., and Gilman, M. Z. (1993) Genes & Dev. 7, 11779–11783
10. Syder, U. K., Thompson, J. F., and Landy, A. (1989) Nature 341, 252–254
11. Syder, U. K., Thompson, J. F., and Landy, A. (1989) Nature 341, 252–254
12. Syder, U. K., Thompson, J. F., and Landy, A. (1989) Nature 341, 252–254
13. Syder, U. K., Thompson, J. F., and Landy, A. (1989) Nature 341, 252–254
14. Syder, U. K., Thompson, J. F., and Landy, A. (1989) Nature 341, 252–254
