The human T-cell leukemia virus type I Tax protein forms a ternary complex on DNA in association with a host factor, the cyclic AMP response element-binding protein (CREB). An understanding of the precise geometry of this complex has been elusive. We have used photocross-linking to investigate Tax-DNA contacts. Our data show that Tax contacts the DNA at two symmetric positions 14 nucleotides apart on either side of the Tax responsive element. The presence of symmetric, widely separated regions of contact suggests that at least two molecules of Tax are present in the complex. Mapping the contacts onto a three-dimensional model of the CREB-DNA binary complex shows that they lie on the same face of the DNA near the regions where the N termini of the CREB bZIP domains enter the major groove. This location correlates well with previous evidence that CREB amino acid residues immediately N-terminal to the bZIP domain are crucial for the formation of the ternary complex. The limited number of cross-links observed suggests that contacts are primarily with the phosphate backbone and does not support the idea that a major structural element of the Tax protein inserts into the major or minor grooves of the DNA.

Human T-cell leukemia virus type I is the causative agent of adult T-cell leukemia/lymphoma and HTLV-I-associated myelopathy (reviewed in Refs. 1 and 2). As in all retroviruses, HTLV-I RNA synthesis is under the control of a promoter located in the proviral long terminal repeat. The HTLV-I-encoded Tax protein is crucial for the regulation of viral RNA synthesis. Tax binds in association with a host factor, CREB, to Tax-responsive elements located in the proviral promoter (3–10). The presence of the Tax-CREB complex significantly increases transcription from the proviral promoter in vitro and in vivo (4, 5, 11–14).

Electrophoretic mobility shift assays demonstrate that the 40-kDa Tax and the 35-kDa CREB proteins form a ternary complex with the TxRE DNA (4–10, 13, 15). The TxRE consists of a nearly symmetric, central 8-base pair cyclic AMP response element flanked on both sides by G-rich and C-rich DNA sequences (3, 7–9, 13, 16). CREB recognizes the CRE through a specific interaction of the bZIP domain of the protein with functional groups in the major groove of the DNA. The binding of Tax is less well understood but is known to require the DNA sequences that flank the CRE (3, 4, 8, 9, 13). These flanking sequences are also required for Tax-dependent transcriptional activation in vivo (4, 13, 16). Tax stabilizes the CREB-DNA complex and induces a small, characteristic supershift in electrophoretic mobility (3, 4, 6, 10, 13, 15). The effect of Tax on the complex stability is thought to be attributable, in part, to the stabilization of the CREB dimer and in part to enhancement of protein-DNA interactions (3, 4, 17, 18).

The precise geometry of the Tax-DNA complex is not known. Previous studies to define the nature of the Tax-CREB-DNA complex have focused primarily on mutational analysis of the protein and DNA components. There is a need to complement these studies with biochemical and biophysical approaches to help map the three-dimensional arrangement of the components of the complex. Currently, it is not clear which face of the DNA helix is contacted by Tax, whether elements of Tax insert into the major or minor grooves of the DNA, whether Tax binds the DNA in a symmetric or asymmetric manner, or even if there is any actual contact between the Tax and the DNA at all. In this paper, we seek to explore the geometry of the Tax-CREB-DNA complex using photocross-linking with aziridine and azidopirimidine-containing DNA.

Aziridine-based cross-linking has been widely used to study protein-nucleic acid interactions (reviewed in Ref. 19). Upon exposure to ultraviolet light, the azirine forms a nitrene that reacts relatively nonspecifically with nearby proteins to form a covalent adduct (19). In our experiments, the azirine group was introduced into the backbone of DNA at specific sites by reaction of 4-azidophenacyl bromide with monophosphorothioate-containing DNA (20–23). The azirine can react with functional groups within a 9 Å radius of its attachment point (20), which allows the azirine to react with both minor groove binding proteins and major groove binding proteins.

We also performed photocross-linking assays using aziridinyl-substituted DNA (19, 24). The nucleotide analogs, 5-iodouridine and 5-iodocytidine, contain an iodine atom that lies in the major groove of the DNA. Because the iodine is bonded directly to the pyrimidine ring, rather than to a flexible linker, it detects only short range contacts with major groove binding proteins. The chemistry of this cross-linking reaction is also believed to be somewhat more selective than that of azirines (19, 24).

In the present study, we used 40 different DNA probes, each substituted with a photoreactive group at a different position. We identified two regions of the DNA backbone that appear to...
contact Tax. One of these regions is present in each half-site of the nearly symmetric TxRE, on the “bottom” face of the DNA as conventionally viewed, very near the point at which the bZIP domain of CREB enters the major groove of DNA. The limited extent of the DNA in contact with Tax argues against the insertion of a major secondary structural element of Tax into either the major or minor grooves of DNA.

MATERIALS AND METHODS

Protein Purification—TaxH6 protein was expressed in Escherichia coli as described (25). The bacterial pellet from a 12-liter culture was resuspended in 40 ml of buffer containing 100 mM Tris-HCl, pH 8.0, 100 mM KCl, 10 mM 2-mercaptoethanol, 1 mM PMSF, and 10 mg/ml lysozyme. The suspension was incubated for 30 min at room temperature and sonicated at 4 °C. The lysate was centrifuged at 12,000 × g for 30 min at 4 °C. The supernatant was adjusted to 1 M ammonium sulfate and incubated for 2 h at 4 °C, and the resulting precipitate was collected by centrifugation at 17,000 × g for 15 min. The pellet was resuspended in 10 ml of 50 mM KPO4, pH 8.0, 500 mM KCl, 4 mM 2-mercaptoethanol, 1 mM PMSF, and 5 mM imidazole and was tumbled for 2 h with 6 ml of Ni+-nitrilotriacetic acid-agarose resin (Qiagen). The resin was poured into a chromatographic column and washed with 120 ml of a buffer containing 50 mM KPO4, pH 7.0, 300 mM KCl, 4 mM 2-mercaptoethanol, 1 mM PMSF, and 25 mM imidazole. The column was developed with a linear gradient of 50 mM KPO4, pH 7.0, 300 mM KCl, 4 mM 2-mercaptoethanol, 1 mM PMSF, and 25 mM imidazole to 50 mM KPO4, pH 8.8, 300 mM KCl, 4 mM 2-mercaptoethanol, 1 mM PMSF, and 500 mM imidazole. Tax eluted at approximately 300 mM imidazole. Tax-containing fractions were dialyzed into a buffer containing 25 mM Hepes, pH 7.9, 150 mM KCl, 1 mM EDTA, 10% glycerol, and 4 mM 2-mercaptoethanol and were stored at −80 °C. CREB A protein was expressed in E. coli and purified by heparin-agarose chromatography as described (26).

Binding Reactions—Binding reactions contained 100 nm CREB, 0 or 300 nm Tax, 520 µM DNA, 5.2 µg/ml poly(dI–dC), 12.5 µM Hepes, pH 7.9, 75 mM KCl, 0.4 mM EDTA, 2.5 mM MgCl2, 1 µM ZnSO4, 6% glycerol, 0.6 mM 2-mercaptoethanol, and 0.2 µg/ml PMSF in a final volume of 20 µl. The DNA probe was a 24-base pair fragment containing the most promoter-proximal of the three TxREs present in the HTLV-I promoter (for sequence, refer to Fig. 1). Reactions were incubated for 15 min at room temperature, and binding was analyzed by 5% PAGE using a Tris-glycine running buffer (3). Results were quantitated by PhosphorImager analysis.

Azido-modified Oligonucleotides—Oligonucleotides containing a single phosphorothioate modification were obtained from Cybersyn (Lenni, PA). Oligonucleotides were annealed to their unmodified complements and gel purified. The resulting double-stranded oligonucleotides were labeled by incubation with [γ-32P]ATP and T4 polynucleotide kinase. They were then incubated for 3 h in the dark at room temperature in a reaction containing 65–75 nm oligonucleotide, 40 mM KPO4, pH 7.0, 60% MeOH, and 12 mM 2-azidothymidine bromide (22). Azido-modified oligonucleotides were isolated by extraction with phenol two times and precipitation with ethanol (20). Purified oligonucleotides were incubated with Tax and CREB under standard binding conditions and exposed to 302 nm ultraviolet light (AlphaImager 2000, Alpha Innotech, San Leandro, CA) for 1 min. Short wavelength light (<300 nm) was blocked by use of a polystyrene multivell plate (19, 23). The mixtures were analyzed by 10% SDS-PAGE, and cross-linked products were detected by PhosphorImager analysis.

Iodopyrimidine-substituted Oligonucleotides—Oligonucleotides containing a single 5-iodo-d-2′-deoxyuridine or 5-iodo-d-2′-deoxycytidine were obtained from Cybersyn (Lenni, PA) and DNA Express (Fort Collins CO). The oligonucleotides were annealed to their unmodified complements, gel-purified, labeled, incubated in a binding reaction, and cross-linked as described above.

Immunoprecipitations—Immunoprecipitation was performed in order to distinguish cross-linked Tax-DNA complexes from CREB-DNA complexes. The following antibodies were used: anti-Tax, monoclonal antibody 1312 (NIH AIDS Reference Reagent Program); anti-CREB, polyclonal rabbit serum directed against amino acids 1–205 (Upstate Biotechnology, catalog number 06-504); and a control antibody, monoclonal antibody 8WG16 directed against RNA polymerase II C-terminal domain (26). Antibodies were mixed with protein G-agarose beads (Sigma) at approximately 2 µg IgG/µl beads. The beads were allowed to bind antibody overnight at 4 °C and then were washed three times with a buffer containing 10 mM Tris, pH 8.0, 500 mM NaCl, and 0.1% Nonidet P-40. Protein-DNA binding reactions were assembled on a preparative scale (final volume, 200 µl), incubated, and irradiated with UV light as described above. Aliquots were removed (40 µl) and mixed with 60 µl of buffer containing 16 mM Tris, pH 8.0, 800 mM NaCl, and 0.16% Nonidet P-40. This mixture was incubated with antibody-coated protein G beads (25 µl/reaction) for 30 min at 4 °C. The beads were washed three times with buffer containing 50 mM Tris, pH 8.0, 150 mM NaCl, 1% Nonidet P-40, 0.5% deoxycholic acid (sodium salt), and 0.1% SDS. Bound proteins were analyzed by SDS-PAGE.

RESULTS

Design of Oligonucleotide Probes—All of the oligonucleotide probes used in this study contained a 24-nucleotide sequence representing the most promoter-proximal of the three TxREs in the HTLV-I long terminal repeat (7, 16). The central region of the TxRE contains a CRE, which differs from the consensus CRE (5′-TGACGTCA-3′) (27) at one position in the right half-site. Because of this difference, the HTLV-I CRE functions only weakly as a binding site for CREB when Tax is not present (4, 5, 7, 9). In the TxRE, the central CRE is flanked by G-rich and...
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C-rich sequences, as indicated in Fig. 1. These sequences are necessary for interaction with Tax (3, 4, 8, 9, 13).

To generate probes for site-specific cross-linking, oligonucleotides were synthesized with phosphorothioate groups substituted for backbone phosphate groups at each of 20 individual positions (Fig. 1A). The numbering scheme is based on that of Ellenberger et al. (28) and is explained in the figure legend. Phosphorothioates are chiral, and all probes are a mixture of the two stereoisomers. To generate the photoreactive probes, phosphorothioate-containing DNA was reacted with 4-azidophenacyl bromide as described under “Materials and Methods.” Oligonucleotides were also synthesized with iodouridine or ido-cytidine substituted at various positions. These are diagrammed in Fig. 1B.

Binding of Tax and CREB to Modified Probes—The binding of CREB and Tax to the 24-nucleotide TxRE-containing DNA was measured in an electrophoretic mobility shift assay. Because the ultimate goal of our experiments was to prepare cross-linked adducts in high yield, binding reactions were performed using a relatively high concentration of CREB (100 nM). This was sufficient to give about 25% saturation of wild-type, unsubstituted probe in the absence of Tax (Fig. 2A). When Tax was added, there was a small supershift in the mobility of the CREB-DNA complex, which is typical for this system and is indicative of ternary complex formation (3, 4, 6, 10, 13, 15). The addition of Tax also increased the fraction of DNA that was bound by protein. The increase was small (about 1.5-fold) as expected under conditions of high CREB concentration (3).

Each of the modified probes was tested in this assay for the ability to form a binary CREB-DNA complex and a ternary Tax-CREB-DNA complex. Although there was some variation in binding efficiency, none of the probes differed from the unmodified DNA by more than 3-fold (Fig. 2, compare A and B). In many cases, the variation is likely to be within the range of experimental error. The addition of the aryl azide group did not appear to affect binding, because the fraction of DNA bound was approximately the same before and after reaction with 4-azidophenacyl bromide (Fig. 2, compare B and C). These results suggest that none of the phosphate modifications disrupts a contact that is, in itself, essential for protein binding. We note, however, that because the protein concentrations approached saturation, small changes in binding affinity might not have been detected.

Conditions for Aryl Azide Cross-linking—We next examined the ability of the aryl azide-containing probes to form covalently cross-linked adducts with protein. Complexes were formed, irradiated with UV light, and analyzed by SDS-PAGE to detect covalently cross-linked products. Fig. 3 shows a representative result. The probe used in this experiment was derivatized at P7L. No cross-linked product was formed in the absence of the aryl azide modification (lanes 1 and 2). In the presence of the aryl azide modification, exposure to ultraviolet light for 1 min induced the formation of a prominent protein-DNA adduct that migrated at approximately 60 kDa (lanes 6 and 7). The amount of the cross-linked species increased in the presence of Tax, but the mobility in SDS-PAGE remained the same (compare lanes 6 and 7). Subsequent experiments showed that this band was a mixture of CREB-DNA and Tax-DNA adducts (see below). Tax and CREB are similar in size (40 and 35 kDa, respectively), and the two proteins do not resolve well in SDS-PAGE. The formation of covalently cross-linked products was dependent on exposure to UV light (compare lanes 6 and 7 with lanes 4 and 5). In the absence of CREB, Tax did not cross-link to the DNA (lane 3), consistent with previous studies showing that Tax does not bind DNA in the absence of CREB or other cellular proteins (5, 13). Exposure to UV light for times longer than 1 min did not increase the yield of cross-linked products (data not shown). Consistent with this, UV spectrophotometry showed that the unreacted azide, which absorbs strongly at 300 nm, was eliminated after 1 min of irradiation under the conditions used for cross-linking (data not shown).

Use of Aryl Azide Probes to Map Protein-DNA Contacts—In order to map the spatial location of contacts between Tax, CREB, and DNA, each of the 20 azido-modified probes was used in the cross-linking assay (Fig. 4). The aryl azide cross-linkers can react with functional groups within a 9 Å radius of
Use of Iodopyrimidine-substituted Probes to Map Protein-DNA Contacts—Whereas the aryl azide probes detect long range (9 Å) contacts in the vicinity of the phosphate backbone, iodopyrimidine-substituted DNA detects short range contacts in the major groove. Based on studies with model systems, the reaction is thought to occur preferentially with aromatic and certain other amino acid residues (19).

We synthesized 20 probes that were substituted with iodopyrimidines at individual positions (Fig. 1). Each of these probes was tested in an electrophoretic mobility shift assay to discover whether the iodopyrimidine substitution affected the ability to form a Tax-CREB-DNA complex. The results are shown in Fig. 5A. None of the iodopyrimidine substitutions outside the CRE significantly affected protein binding. However, there were 4 or 5 probes with iodopyrimidine substitutions in the central part of the CRE that showed a somewhat reduced ability to form both CREB-DNA and Tax-CREB-DNA complexes. These substitutions are in a region where there is believed to be extensive, close contact between CREB and functional groups in the major groove, and it may be that addition of the iodine atom to

Their attachment point (20). Protein-DNA complexes were formed with and without Tax, and the reactions were immunoprecipitated using an anti-Tax and an anti-CREB antibody to distinguish the Tax-DNA and the CREB-DNA adducts. Control reactions were also done using an unrelated antibody (anti-

RNA polymerase II). For comparison, an equivalent amount of each binding reaction, prior to immunoprecipitation, is also shown.

Two probes, P7L and P7R, gave prominent bands when the reaction products were immunoprecipitated with the anti-Tax antibody. We classified P7L and P7R as positions where Tax closely approaches DNA, based both upon the amount of the cross-linked Tax-DNA adduct, relative to other probes, and on the fact that the Tax-DNA adducts were produced in greater yield than the CREB-DNA adducts. The P7L and P7R positions are symmetrically related in opposite half-sites of the TxRE (Fig. 1). These positions lie in the center of the four-nucleotide, G-rich flanking sequences that have previously been shown to be required for the interaction of Tax with the TxRE.

Another probe, P1’L, cross-linked somewhat more weakly to Tax. The P1’L position lies on the same face of the helix as P7L but is separated by the width of the major groove. The cross-link at this position was seen only in the left half-site.

Although the primary purpose of these experiments was to map Tax-DNA contacts, they also convey some information about the geometry of CREB-DNA contacts. All of the probes showed some reactivity with CREB, perhaps because the high concentration of CREB in the assays allowed some nonspecific DNA binding. However, some probes showed reactivity that was significantly above background. There was one pair of probes, P3L and P3R, where strong CREB-DNA cross-links were present in both half-sites. Surprisingly, there are a number of positions where cross-links were somewhat stronger in one half-site than in the other. These include contacts at P9R, P7R, P5L, P1L, and P1’L, which were stronger than the contacts at the symmetry-related positions, P9L, P7L, P5R, P1R, and P1’R. Although some of these differences may reflect inherent variability in the assay, there does appear to be an underlying pattern reflective of a small concerted shift in the relative position of CREB and DNA in the two half-sites (see “Discussion”). This minor shift in the position of CREB is presumably needed to accommodate the asymmetry in the DNA sequence caused by the presence of a nonconsensus base pair in the right half-site.

In some cases, the efficiency of CREB-DNA cross-linking is increased in the presence of Tax. This is most evident at P7R but is seen to some extent at P7L and P3R. These increases may be attributable to local conformational changes, because the efficiencies of the other CREB-DNA cross-links do not change in the presence of Tax.

Fig. 4. Immunoprecipitation of Tax and CREB cross-linked to the azido-modified oligonucleotides. Cross-linking reactions were carried out with or without Tax as described under “Materials and Methods.” Reaction products were immunoprecipitated using anti-Tax (Tax), anti-CREB (CREB), or an unrelated control antibody (Ctrl) as indicated. An equivalent amount of each binding reaction prior to immunoprecipitation is also shown and is indicated with a dash. The figure is a composite, with intensities in different panels scaled to compensate for differences in probe specific activity and exposure time.
cytosine (or, in one case, substitution of the iodine for the methyl group in thymidine) perturbs the interaction of CREB with the DNA.

The results of cross-linking experiments using the iodopyrimidine-substituted probes are shown in Fig. 5B. The Thy-4L and Thy-4R probes cross-linked weakly to Tax. However, the signal was only slightly above the background seen when the control, CREB-only reaction was precipitated with anti-Tax antibody. The other probes showed no detectable reactivity with Tax.

The Thy-4L and Thy-4R probes also cross-linked strongly with the CREB protein. Model building, based on the structure of the closely related GCN4 protein, predicts that the methyl groups of Thy-4L and Thy-4R are in close contact with Cys286 (see “Discussion”). Cysteine is known to cross-link to bromopyrimidines and is thought to cross-link to iodopyrimidines in a similar manner (19, 29). Thus, the presence of a cross-link between CREB and DNA at this position is consistent with structural data. The probes with iodopyrimidine substitutions in the center of the CRE showed surprisingly little ability to form cross-links to either CREB or Tax. This result may be attributable, in part, to steric interference with binding caused by the iodine atom. Also, the CREB amino acids nearest to these positions in the DNA (Asn279 and Arg287) may be inherently less reactive than cysteine (19).

**DISCUSSION**

The experiments presented here address a long standing problem in the HTLV-I field, which is the position of the Tax protein in the Tax-CREB-DNA complex. We prepared a set of aryl azide-containing probes in which every other phosphate group was derivatized on each strand of the DNA over a 17-nucleotide region containing an HTLV-I-derived TxRE. Two additional phosphate groups at the center of the TxRE were also derivatized. Because each aryl azide group can react with protein within a 9 Å radius of its attachment point, these probes should provide complete coverage of the full TxRE. Cross-linking experiments using these probes show that Tax contacts the DNA at two symmetric positions 14-nucleotide apart on either side of the TxRE (positions P7L and P7R). The presence of two symmetric, widely separated regions of contact strongly suggests that at least two molecules of Tax are present in the Tax-CREB-DNA complex.

To help visualize which face of the DNA helix is contacted by Tax, we mapped the cross-linking data onto a three-dimensional model of the CREB-DNA binary complex. The model is based on the crystal structure of GCN4, a related bZIP protein, bound to a CRE-containing DNA (30). Although CREB and GCN4 differ in primary sequence, key structural elements are conserved, including the dimerization interface and the amino acids that make base-specific contacts with DNA. Several views of the final model are shown in Fig. 6. The P7L and P7R positions, which strongly cross-link to Tax, lie on the opposite face of the DNA from the CREB dimerization interface, near where the bZIP domain of CREB enters the major groove (Fig. 6, B and C). A weaker Tax-DNA cross-link at P19L lies across the major groove from P7L, which is also near where the bZIP domain of CREB enters the major groove. All three of these probes, P7R, P7L, and P19L, also react with CREB, a finding that is consistent with the position of CREB in the model. Taken as a whole, the pattern of cross-linking suggests that the P7L and P7R positions lie at a nexus where the three macromolecular components of the complex, Tax, CREB, and DNA, exist in close proximity.

The present results are in striking harmony with the results
FIG. 6. A model of the CREB-DNA binary complex based on the GCN4-DNA crystal structure. The structure is based on Ref. 30. A, primary sequence of the CREB bZIP domain. Residues 273–327 are included in the structural model. Residues involved in base-specific protein-DNA contacts are also shown in bold and underlined. The hydrophobic 4,3 repeat is indicated below the sequence using the conventional “abcdefg” nomenclature (36). Residues at the conserved hydrophobic a and d positions are shown in bold and underlined. The boxed residues indicate the residues crucial to the formation of the Tax-CREB-DNA ternary complex identified by Adya et al. (31). B, front view of structural model illustrating the CREB-DNA binary complex. The model was constructed by replacement of GCN4 amino acid side chain residues with the corresponding CREB residues, by extension of the DNA as a B form helix, and by introduction of necessary sequence mutations in the DNA. The model was solvated and then energy-minimized with the Quanta 97 Charm software package (Molecular Simulations Inc., San Diego, CA). Azido-modified positions that did not cross-link to Tax are indicated in dark blue. Strong cross-links at P7L and P7R are indicated in orange, and a weak contact at P1L is indicated in light orange. Iodine atoms are indicated in dark brown. The remainder of the DNA is indicated in light blue. The two CREB monomers are indicated by pink and purple, respectively. C, same as panel B but a bottom view. D, same as panel A, except that phosphate residues are colored to indicate cross-linking to CREB. Blue, background levels of cross-linking. Green, cross-linking significantly above background. Curved arrows indicate concerted shifts in position of CREB in the right half-site, which are evident from cross-linking data but that are not reflected in model as shown (see text). E, same as panel D but a rear view.
from a previously reported mutational study (31). In that study, an AAR sequence immediately N-terminal to the CREB bZIP domain was substituted with a PQL sequence found in ATF-1, another bZIP family member. ATF-1 does not interact with Tax, and substitution of the ATF-1 sequence abolished the ability of CREB to functionally interact with Tax, suggesting that these residues are essential for Tax-CREB interaction. Although these residues lie just outside our CREB-DNA model, they are near the beginning of the bZIP domain and must be reasonably close to the P7L and P7R DNA positions in the native complex.

One of the questions in the Tax field is the extent to which structural elements of the Tax protein insert into the major or minor groove of DNA to make base-specific contacts. To assess whether Tax enters the major groove, we synthesized a set of 20 5'-iodo-substituted probes representing all pyrimidine positions in the relevant portion of the TxE. These probes were negative for cross-linking to Tax, with the possible exception of Thy-4L and Thy-4R, which gave a signal slightly above background (Fig. 5B). Although negative results are not conclusive, these data provide little support for the idea that major structural elements of the Tax protein insert into the major groove. These data also argue against any significant Tax-induced shift of CREB into the region of the major groove that flanks the CRE.

We have not been able to use the photocross-linking approach to probe specifically for minor groove interactions, because to our knowledge, minor groove-specific photocross-linking reagents are not available. We note, however, that if a significant structural element of Tax were inserted into the minor groove, one would expect that more cross-links would be seen with the aryl azide probes, including sets of cross-links located across the minor groove from one another. Although there may be sporadic Tax side chain contacts in the minor groove, the data argue against the presence of an extensive interaction.

Although the purpose of our study was to use photoreactive probes to map the position of Tax in the Tax-CREB-DNA complex, the aryl azide cross-linking data also reveal some information about CREB-DNA contacts. The probes that we have used, which are based on the natural HTLV-I sequences, do not contain a perfect match to the consensus CRE. There is an AT base pair, instead of the consensus TA, at the 2R-29 position (adjacent to phosphates P1R and P1′R). Careful inspection of the CREB-DNA cross-linking pattern suggests that this causes a slight, concerted motion of CREB relative to DNA in the two half-sites (denoted by the curved white arrows in Fig. 6, D and E). This is reflected in the weakening of some cross-links in the right half-site and the strengthening of others. Generally, these alterations involve pairs of phosphates located on opposite sides of the major groove. Thus, there is a weakening of cross-linking at P5R and a strengthening at P1L. There is also a weakening at P1′R and a corresponding strengthening at P7R and P9R. An overall shift in the position of CREB in the right half-site probably explains the absence of the Tax cross-link at P1′R, whereas a weak Tax cross-link was present at P1′L.

On a cautionary note, it is of interest that we detected only weak-to-moderate CREB-DNA cross-linking at the P0 and P0′ positions. The phosphates at these positions are believed to make weak contacts with CREB. Model building suggests that the side chains of residues Arg286 Arg284 and Arg287 all approach within 5 Å or less of the P0 and P0′ phosphates, well within the range of the aryl azide cross-linker. It may be that the contacts at P0 and P0′ are so extensive as to force the aryl azide moiety to rotate away from the CREB, where it is less able to react. In any case, the failure to obtain efficient cross-linking at these positions illustrates the fact that false negative results are sometimes obtained with the aryl azide method.

There is considerable evidence that Tax functions as a dimer in vivo (32–34). Our cross-linking data are consistent with this in that they suggest that there are two Tax molecules present in the Tax-CREB-DNA complex, one in each half-site. However, this finding raises the question of where the Tax dimerization interface is located, relative to the CREB and DNA. The most plausible suggestion is that it threads symmetrically between the two CREB monomers, across the bottom of the complex, and 180° around the DNA helical axis from the CREB-CREB dimerization interface. Because we did not detect Tax-DNA cross-links at positions P1′R, P3′L, and P3′R, which lie in this region, we suspect that the Tax-Tax interface, if present, may lie some distance removed from the surface of the DNA.

The cross-linking experiments do not address the location of the remaining 273 amino acids of CREB that lie N-terminal to the bZIP domain. Presumably, these residues lie, at least in part, opposite the CREB-CREB dimerization interface (i.e. on the bottom surface of the complex as conventionally viewed). Only one probe, P9R, showed a strong cross-link to CREB at a position remote from the bZIP domain. This contact is on the bottom face of the DNA helix and provides a clue that a missing portion of the CREB protein may lie in this vicinity.

While this manuscript was under consideration, another study appeared that examined the interaction of Tax protein with flanking DNA sequences in the TxE (35). These authors suggest that only a single molecule of Tax is present in the Tax-CREB-DNA complex. However, this study used a smaller number of cross-linking probes that did not provide full coverage of the TxE. This appears to account for the difference in results.

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