Quantitative studies of the effect of HTLV-I Tax protein on CREB protein – DNA binding

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ABSTRACT

The human T-cell leukemia virus type I (HTLV-I) Tax protein increases the DNA binding activity of a number of different host cell transcription factors, including the cyclic AMP response element binding protein (CREB). We have performed quantitative studies of CREB binding in the presence and absence of Tax in an attempt to gain insight into the mechanism of the Tax effect. Enhancement of binding occurred over a wide range of CREB concentrations, but sharply increased at the lowest concentrations tested. The data are best explained by a two-step binding model where Tax changes the apparent equilibrium constants for both a CREB – CREB dimerization step and a (CREB)₂ – DNA binding step. We used the model to perform a quantitative analysis of the binding of CREB to DNA that had been mutated at positions flanking the core CREB recognition site. Results suggest that there are altered or more extensive DNA – protein contacts at these positions in the presence of Tax. We also used the model to analyze differences in the interaction of Tax with nonphosphorylated and protein kinase A-phosphorylated CREB protein. There was no significant change in the behavior of CREB upon phosphorylation.

INTRODUCTION

Human T-cell leukemia virus type I (HTLV-I) is an oncogenic retrovirus that causes adult T-cell leukemia and several neurological disorders (reviewed in (1)). The virally encoded Tax protein is important both for viral replication and for viral pathogenesis. Tax interacts with the host cell transcription machinery and stimulates transcription of HTLV-I RNA by host cell RNA polymerase II (2–8). In addition, Tax increases the transcription of a number of cellular genes, some of which are involved in the control of cell growth, including IL-2, IL-2 receptor α subunit, GM-CSF, and fos (9–16). This effect of Tax on cellular gene expression may contribute to neoplastic transformation (17, 18). Tax also decreases transcription of the DNA polymerase β gene, and by doing so may interfere with normal DNA repair (19).

Genetic studies show that Tax activates transcription through DNA sequences called Tax responsive elements (TxREs). TxREs

have been defined both in the HTLV-I proviral promoter and in Tax-responsive cellular promoters. The TxREs in the HTLV-I promoter coincide with binding sites for transcription factors in the CREB/ATF family, and biochemical studies have shown that purified Tax protein alters and/or enhances the DNA-binding activity of CREB, ATF-1, and ATF-2 to these sites (20–26). Purified Tax increases transcription of the HTLV-I promoter in an *in vitro* reaction (21), and this effect is enhanced in the presence of exogenous CREB (24).

Interestingly, the effect of Tax on host cell protein–DNA interactions is not limited to the CREB/ATF family. TxREs in cellular promoters have been found to coincide with binding sites for serum response factor, NF- κ B, and members of the fos/jun family (13, 27–31). *In vitro* studies have shown that the DNA binding activity of each of these factors is increased by Tax (23). In addition, Tax stimulates the binding of the structurally unrelated proteins, Sp1 and GAL4 (DNA binding domain) (23). This broad *in vitro* specificity is consistent with the pleiotropic effects of Tax on *in vivo* gene expression.

The effect of Tax on the binding of host cell proteins does not require the energy of ATP hydrolysis, and does not appear to involve oxidation–reduction (21, 23). It has been suggested that the effect on ATF-2-DNA binding involves stabilization of protein dimers (26), or alternatively, that the interaction with CREB involves a Tax–CREB–DNA ternary complex (20, 22, 25). Quantitative studies of the Tax effect, which might give insights into the underlying mechanism, have not yet been reported. In the present study, we put forward a quantitative model to explain the effect of Tax on CREB–DNA binding interactions. The model provides a conceptual framework within which one can begin to assess the effect of mutations in recognition sites, target proteins, and Tax itself. We find that the fold activation of binding by Tax is very sensitive to reaction conditions, and we suggest that changes in apparent equilibrium constants provide a more reliable measure of the Tax effect.

MATERIALS AND METHODS

Protein purification

The *E. coli*-expressed Tax protein used in most experiments contained a histidine tag at the C-terminus (20). This protein (TaxH₆) was purified by Ni²⁺ NTA-agarose and gel filtration

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chromatography as described (32). Baculovirus-expressed Tax protein, used where indicated in Figure Legends, was purified as described (21). CREB A protein was expressed in *E. coli*, purified by the boiling method (33), and subjected to gel filtration chromatography (32). For some CREB preparations (a gift from M. Matthews), heparin-agarose chromatography was substituted for gel filtration. The CREB was applied to the heparin agarose column in a buffer of 50 mM 4-(2-Hydroxyethyl)-1-Piperazineethanesulfonic Acid (HEPES), 1.0 mM [Ethylenedinitrilo]-Tetraacetic Acid Disodium Salt (EDTA), 20% glycerol, 1 mM dithiothreitol, and 10 μ g/ml Phenylmethylsulfonyl Fluoride (PMSF), and washed with two column volumes of the above buffer plus 0.1 M KCl. Bound CREB was eluted from the heparin-agarose column by a linear gradient of KCl from 0.1 M to 1.1 M KCl over ten column volumes. Peak fractions eluted at about 0.5 M KCl.

Binding reactions

Reactions were carried out as described (32) and contained either 8 μ l of HE buffer (25 mM HEPES pH 7.9 at 25°C, 150 mM KCl, 1 mM EDTA, 0.1% Nonidet P-40 (NP-40), 10% glycerol, 4 mM 2-mercaptoethanol (2-ME), and 10 μ g/ml PMSF) or 8 μ l of Tax protein at 100–200 nM final concentration in HE buffer, 5 μ g/ml poly (dI-dC), 2 μ l of CREB protein diluted in HMZ buffer (25 mM HEPES, 150 mM KCl, 12.5 mM MgCl₂, 10 μ M ZnSO₄, 4 mM 2-ME, 0.1% NP-40, 10 μ g/ml PMSF, and 20% Glycerol), and 0.4–0.5 nM DNA; brought to a final volume of 20 μ l with H₂O. The DNA probe was an 85 basepair, gel-purified restriction fragment containing the most promoter proximal HTLV-I TxRE (24). Reaction products were electrophoresed in a 5% non-denaturing polyacrylamide gel (39:1 acrylamide:N,N-methylene bisacrylamide) in a Tris–Glycine–NP-40 gel buffer system (32). DNA complexes were visualized by autoradiography and phoshoImage analysis.

Models for CREB–DNA binding

Two models were tested for their ability to explain the experimental data. In these expressions, P represents monomer CREB, and D represents a DNA fragment containing a single, partially dyad-symmetric CREB DNA recognition site. Data was initially fitted using a simple association model, by the procedure described previously (34).

Because the simple association model could not adequately explain the effect of Tax, data was also fitted using the multi-step binding model. A complete description of the binding of a dimeric protein to DNA is given by the expression:

To facilitate analysis, we have assumed that the monomer CREB–DNA complex (P–D) does not significantly contribute to the reaction, so that a simplified expression may be written:

The dissociation constants K_1 , and K_2 can be defined in terms of the concentrations of the species in the reaction as follows:

$$K_1 = \frac{[P]^2}{[P_2]} \quad (4)$$

$$K_2 = \frac{[P_2][D]}{[P_2 - D]} \quad (5)$$

The fractional saturation of the DNA probe may be defined as

$$\theta = \frac{[P_2 - D]}{[D] + [P_2 - D]} \quad (6)$$

Substituting equations (3), (4), and (5) into (6) and rearranging gives the the following:

$$\theta = \frac{[P]^2}{K_1 K_2 + [P]^2} \quad (7)$$

We assume that since free protein is in great excess over DNA-bound protein, free monomer concentration is determined principally by the monomer-dimer equilibrium:

Free protein concentration can therefore be related to input protein, P_{tot} , as follows:

$$[P_{\text{tot}}] \approx [P] + 2[P_2] \quad (9)$$

Rearranging and substituting from equation [4], one obtains a quadratic,

$$0 + \left(\frac{-2}{K_1} \right) P^2 - P + P_{\text{tot}} \quad (10)$$

and applying the quadratic formula, one obtains the expression

$$P = \frac{K_1 \left(1 - \sqrt{1 + \frac{8[P_{\text{tot}}]}{K_1}} \right)}{-4} \quad (11)$$

which can then be substituted in equation (7) in order to obtain an expression for the fraction of DNA bound as a function of P_{tot} , the independent variable, and the parameters K_1 and K_2 . Optimal values for K_1 and K_2 were determined by nonlinear least squares regression analysis using the Igor program (WaveMetrics, Lake Oswego, OR).

Changes in CREB–DNA binding in the presence of Tax

Tax presumably associates with one or more of the complexes in [2]. This association may be quite weak, consistent with the 100–200 nM Tax concentrations required to see an effect, and therefore difficult to measure directly. In the experiments reported

here, we have not attempted to measure the association of Tax and CREB directly, and we have not explicitly included the binding of Tax in the reaction scheme. Rather, we have measured CREB–DNA binding in the presence of Tax. From these data, we have determined changes in the apparent K_1 and K_2 induced by Tax.

RESULTS

Experimental observations

The studies reported here used Tax made in a baculovirus expression system (21), or a histidine-tagged Tax (TaxH₆) derivative expressed in *E. coli* (20). CREB A was expressed in *E. coli* (33). Proteins were purified as described in Materials and Methods. An SDS–PAGE analysis of the purified proteins is shown in Figure 1A.

An electrophoretic mobility shift assay using these purified proteins is shown in Figure 1B. The radiolabeled DNA probe contained a single TxRE (Materials and Methods). CREB bound, apparently as a dimer, to the single partially dyad-symmetric recognition site in this fragment, forming a discrete CREB₂–DNA complex. In the presence of Tax, the amount of this complex was increased and its migration in the gel was slightly retarded. Preliminary time course experiments indicated that the binding had reached equilibrium in a standard 12–15 min. reaction (not shown). Previous competition experiments (21, 23, 24) indicated that the binding of CREB is sequence specific in both the absence and presence of Tax.

Figure 1. (A) Proteins used in DNA binding reactions were analyzed by 10% SDS–PAGE, and visualized by silver staining (43). Lane 1, molecular weight standards, 66.2, 45.0, 31.0, and 21.0 kDa, top to bottom. Lane 2, CREB purified by heparin-agarose method, lane 3, TaxH₆, lane 4, Tax from baculovirus expression system. Left arrow indicates CREB, right arrow indicates TaxH₆. (B) Electrophoretic mobility shift assays. Reactions contained radiolabeled HTLV-I TxRE DNA and 0.74 nM CREB purified by heparin-agarose method. Lane 1, reaction without Tax, lane 2, reaction with 0.2 μM TaxH₆. Left arrow indicates CREB₂–DNA complex, right arrow indicates CREB₂–DNA complex in the presence of Tax, which is slightly retarded in mobility. Free DNA probe is at bottom of gel.

In order to gain a more quantitative understanding of the effect of Tax, the concentration of CREB was varied in the presence and absence of Tax, and DNA binding was measured in the electrophoretic mobility shift assay. The fraction of the DNA that was bound to protein was quantitated by PhosphorImager analysis and expressed as a function of CREB concentration (Figure 2A). The same data have been replotted in Figure 2B

Figure 2. CREB–DNA binding in the presence and absence of TaxH₆. (A) Fraction of DNA bound in the absence (open squares) and the presence of 0.2 μM TaxH₆ (closed squares) as a function of CREB concentration. Solid lines were determined by fitting the data using a cooperative binding model. Parameters in absence of TaxH₆ were $K_1=7$ nM, $K_2=60$ nM, and in the presence of TaxH₆ were $K_1=0.1$ nM, $K_2=14.4$ nM. (B) Data from panel A replotted to show fold stimulation of binding by Tax, defined as fraction of DNA bound in presence of TaxH₆ divided by fraction of DNA bound in the absence of TaxH₆ (closed squares). The fitted binding curves from (A) were used to generate a theoretical fold stimulation curve (solid line). A theoretical fold stimulation curve (dotted line) was also generated using a one-step, non-cooperative association model (see Materials and Methods). (C–F) Effect of varying individual binding parameters. (C) Lower curve identical to lower curve in panel A, upper curve generated by decreasing K_1 to 0.1 nM and holding K_2 constant. (D) Fold stimulation calculated from binding curves in panel C. (E) Lower curve identical to lower curve in panel A, upper curve generated by decreasing K_2 to 14.4 nM and holding K_1 constant. (F) Fold stimulation calculated from binding curves in panel E.

to show fold-stimulation by Tax, defined as the fraction of DNA bound in the presence of Tax divided by the fraction of DNA bound in the absence of Tax.

The stimulation curve in Figure 2B has a distinctive shape. Although stimulation was observed at all concentrations of CREB, the effect of Tax increased sharply at the lowest CREB concentrations tested. The fold-stimulation by Tax becomes difficult to measure precisely at the lowest concentrations of CREB, because the amount of DNA bound in the absence of Tax approaches background values. Thus, considerable variability was found in the numerical values for the fold-stimulation in different experiments. However, the same general pattern of Tax stimulation, dependent on CREB concentration, was seen in all experiments.

Model for CREB–DNA interactions

We developed a quantitative model based on a two-step binding mechanism to account for the CREB–DNA binding data (Materials and Methods). We have assumed that the species that contribute significantly to the reaction are CREB monomer, (CREB)₂ dimer, and (CREB)₂–DNA complexes. For simplicity, we have neglected CREB monomer–DNA complexes (see Materials and Methods).

Apparent dissociation constants for the two steps of binding, K_1 and K_2 , were determined empirically using an iterative fitting procedure (see Materials and Methods). A good overall fit, represented by the lower curve in Figure 2A, was obtained using a dissociation constant for CREB dimers (K_1) of 7 nM. The apparent dissociation constant for (CREB)₂–DNA complexes (K_2) was approximately 60 nM.

Apparent binding parameters in the presence of Tax were also determined by an iterative fitting procedure. The apparent dissociation constant for CREB dimers, K_1 , was decreased. The upper curve in figure 2A reflects a K_1 of 0.1 nM (all values of $K_1 < 0.1$ nM gave similar fits) and an apparent (CREB)₂–DNA dissociation constant, K_2 , of 14.4 nM.

We used the theoretical binding curves, shown as solid lines in figure 2A, to predict the fold-stimulation by Tax as a function of CREB concentration. The results, shown as a solid line in Figure 2B, are in reasonably good agreement with the experimental data and show a marked dependence on CREB concentration. The same data were analyzed using a non-cooperative, one-step binding model (Materials and Methods and, (34)). This model could not account for the sharp increase in Tax effect at low concentrations of CREB, as reflected in the

Figure 3. Nucleotide sequence of wild type and mutant HTLV-I TxREs. Actual HTLV-I sequence is shown on top, and sequences present in DNA probes are shown below. All probes were inserted into the SmaI site of pUC19. The core CREB recognition site is boxed, the mutated sequences within the TxRE are underlined, and pUC19 polylinker DNA starts at the arrowheads. To prepare probes for experiments, a 67 to 75 bp HindIII/ EcoRI fragment was excised from the vector and gel purified.

dashed line of Figure 2B, which does not conform to the experimental data.

Our fitting procedure indicated that Tax caused an apparent change in both K_1 and K_2 . It was of interest to visualize separately the impact of the changes in each binding parameter. Figures 2C and 2D present calculated binding curves where K_1 was decreased in the presence of Tax but K_2 was held constant. The sigmoidal, cooperative binding curve observed in the absence of Tax changes to a shape more like a rectangular hyperbola in the presence of Tax. The change in the shape of the curves, which is most pronounced at low CREB concentrations ($[CREB] \leq K_1$), results in a large stimulation by Tax at low CREB concentrations, but little change in binding at high concentrations.

Figures 2E and 2F present calculated binding data where K_1 was held constant and K_2 was decreased in the presence of Tax.

Figure 4. Analysis of CREB binding to wild type (16.53) and mutant (16.5, 16.3, and 16.0) probes. For graphs (A) and (C–F), data is plotted as an average of two experiments, with error bars indicating standard deviation. Solid lines fit to represent binding curve obtained using the cooperative binding model, as in Figure 2. (A), Fraction of DNA bound in the absence of TaxH₆ as a function of CREB concentration. (B), The fold stimulation in the presence of 0.2 mM TaxH₆. (C–F), Binding of CREB to each of the DNA probes in the absence (open squares) and presence (closed squares) of 0.2 μM TaxH₆.

There is reasonable agreement with the experimental data at high concentrations of CREB, but one does not observe the pronounced increase in the Tax effect at low concentrations of CREB. We conclude from this analysis that changes in both K_1 and K_2 are required in order to account for the experimental observations.

The Tax effect requires sequences flanking the CREB site

We used the model described in the preceding section to perform a quantitative analysis of the binding of CREB to DNA sites that had been mutated at selected positions. Previous workers have noted that the TxREs contained conserved sequences outside the core CREB recognition site, and that mutations in these sequences reduced HTLV-I gene expression *in vivo* (35). It was not clear from the *in vivo* data, however, whether these sequences were important because they interacted directly with CREB or Tax, or because they interacted directly with some additional factor that recognized flanking sequences (see for example, (36, 37)).

To examine the role of flanking sequences in the Tax–CREB–DNA system, we constructed several deletion mutants affecting sequences in the conserved region upstream and/or downstream of the core CREB recognition sequence within the TxRE, as shown in Figure 3. The wild type probe used in these experiments (16.53) contained a full 21 bp HTLV-I TxRE, whereas the mutants lacked TxRE sequences upstream of the core CREB recognition site (16.3), downstream of the core CREB recognition site (16.5), or both (16.0). All four probes bound CREB equivalently in the absence of Tax, consistent with the fact that none of the mutations impinged directly on the core CREB recognition site (Figure 3). However, the four probes showed marked differences in their ability to respond to Tax, as summarized in Figure 4B. Tax strongly stimulated CREB binding with the full TxRE (16.53), had intermediate effects on the 16.5 and 16.3 probes, and had only a minimal effect on the 16.0 probe.

The two-step binding model was then applied to the data (solid lines in Figure 4A, C–F). One would predict that mutations in the TxRE DNA would affect the ability of Tax to influence CREB–DNA interactions (K_2), but would not affect CREB dimerization in solution (K_1). The data are generally consistent with this prediction. The values for K_2 in the absence of Tax ranged from 9–11 nM (the difference from Fig. 2 apparently being attributable to a higher specific activity of the CREB

preparation). Tax decreased K_2 3.8-fold with the wild type probe (16.53), but only 1.7-fold with the mutant probe (16.0). Similar results were obtained with analysis of an independent set of data collected using different preparations of CREB and Tax (not shown). Consistent with the expectation that changes in the DNA should not affect CREB dimerization in solution, we found that we could obtain satisfactory fits when K_1 was constrained to be the same for wild type and mutants and only K_2 was allowed to vary. However, there was too much variability in the data at low concentrations of CREB to be certain whether or not the mutations had an effect on K_1 .

To confirm the finding that flanking sequences are required for the effect of Tax we performed similar experiments using Tax expressed in a baculovirus system. Tax from this source does not contain the C-terminal histidine tag. As shown in Fig. 5, baculovirus-expressed Tax stimulated CREB binding, and the effects were proportionately greatest at the lowest CREB concentrations. Mutations in flanking sequences strongly impaired the ability of Tax to stimulate CREB binding. Not enough data points were collected to derive K_1 and K_2 parameters, but the results appeared qualitatively similar to those obtained with the TaxH₆.

The Tax effect is independent of the CREB phosphorylation state

A previous study showed that phosphorylation of CREB by protein kinase A enhances DNA binding activity in some instances (38). The effect is most pronounced with imperfect CREB sites that are similar, although not identical, to those present in the HTLV-I TxREs. It seemed possible that the effect of Tax and the effect of phosphorylation might involve a common mechanism. That is, Tax and protein kinase A phosphorylation might independently induce a similar conformational change in CREB, favoring the binding to certain DNA sites.

To test this hypothesis, bacterially expressed CREB was phosphorylated by protein kinase A *in vitro*. Previous studies have shown that, under these conditions, phosphorylation occurs at a single site, serine 119 (39). Three sets of incubations were carried out in parallel: mock phosphorylation reactions lacking ATP, phosphorylation reactions containing nonradiolabeled ATP, and separate phosphorylation reactions containing [γ -³²P] ATP to allow determination of the extent of phosphorylation. After

Figure 5. CREB–DNA binding in the presence and absence of baculovirus-expressed Tax. (A) Fraction of wild type (16.53) DNA bound in the absence (open squares) and presence (closed squares) of 0.3 μ M Tax as a function of CREB concentration. (B) As panel A, except that the mutant (16.0) probe was used. (C) Data from Panels A and B replotted to show fold stimulation of binding by Tax.

analyzing the radiolabeled samples to show that phosphorylation approached completion (85%), the other two samples were repurified by gel filtration chromatography to separate CREB from protein kinase A (Figure 6).

The repurified CREB was tested in DNA binding assays. In the absence of Tax, the phosphorylated and mock-phosphorylated samples appeared to bind wild type (16.53) and mutant (16.0) probes identically, within the limits of error (Figure 7A). The phosphorylated and nonphosphorylated CREB were equally responsive to Tax when the wild type probe (16.53) was used (Figure 7B, C). Tax had little effect on the binding of either CREB species to the mutant probe (16.0), as expected (Figure 7D, E). We conclude from these data that Tax and protein kinase A phosphorylation have different and independent effects on CREB.

DISCUSSION

We have performed quantitative studies of CREB–DNA binding in the presence and absence of the HTLV-I Tax protein. We have

Figure 6. Separation of CREB and phosphoCREB from protein kinase A. Phosphorylation reactions were chromatographed on a Superdex 200 HR 16/60 column. Fractions from mock phosphorylation (A) and phosphorylation reactions (B) were assayed for DNA binding activity as in previous experiments. Fractions were assayed for protein kinase A activity by incubating with additional CREB in the presence of [γ - 32 P] ATP and measuring incorporation of radiolabel.

Figure 7. CREB and phosphoCREB DNA binding in the presence and absence of TaxH₆. Data is plotted as an average of two experiments, with error bars indicating standard deviation. Solid lines fit to represent binding curves obtained using the cooperative binding model, as in figures 2 and 4. (A), Fraction of wild type (16.53) and mutant (16.0) probes bound in the absence of TaxH₆, as a function of CREB concentration. (B), Fraction of wild type (16.53) DNA bound by mock-phosphorylated CREB in presence and absence of 0.2 μ M TaxH₆, as indicated. (C), Fraction of wild type (16.53) DNA bound by phosphorylated CREB in presence and absence of 0.2 μ M TaxH₆. (D), As panel B, except mutant (16.0) probe. (E), As panel C, except mutant (16.0) probe.

Figure 8. Model of CREB dimer bound to DNA. Tax is outlined in a dashed line since there is no direct evidence for the stoichiometry and position of Tax in the CREB–DNA complex. Tax associates with CREB, stabilizing dimer formation, and changing the CREB–DNA interactions.

developed a two-step binding model which is in good quantitative agreement with experimental observations. The model allows one to describe the Tax effect in terms of changes in apparent equilibrium constants for CREB dimerization and $(\text{CREB})_2$ -DNA binding. The data suggest that Tax affects both steps in the binding reaction.

The effect of Tax on the dimerization of another bZIP protein, ATF-2, has been previously demonstrated by another group (26). Our data show that the dimerization model is partially applicable to CREB. Our model differs from that of Wagner and coworkers, however, in that the change in dimerization appears to be insufficient to explain the effect of Tax at higher CREB concentrations. To fully account for the data, one must postulate that Tax also has a direct effect on the $(\text{CREB})_2$ -DNA binding interaction (K_2). Consistent with this hypothesis, we have shown that Tax causes CREB to recognize DNA slightly differently. Mutations in sequences flanking the core CREB recognition site had no measurable effect on the binding of CREB by itself, but reduced the ability of Tax to stimulate the binding. The effect was small, but reproducible. While this manuscript was in preparation, another study was published which used binding site selection to establish the importance of flanking sequences for the Tax effect (40). We find it interesting that a relatively small difference in binding energy between wild type and mutant DNA (our quadruple point mutant had only a 2–3 fold change in equilibrium constant, corresponding to less than 1 kcal/mole) is sufficient to give a robust result in the binding site selection assay.

The effect of Tax on $(\text{CREB})_2$ -DNA interactions may be more significant than the effect on dimerization *in vivo*. The nuclear concentration of CREB is estimated to be 400 nM (41). Although our measurements are indirect and subject to error from several sources, all experiments yielded values for K_1 in the absence of Tax between 0.5 nM and 10 nM. Based on this finding, the bulk of the CREB should be present in dimer form *in vivo*. Thus, one predicts that the biological effects of Tax are more likely to result from its action on preformed CREB dimers than from a change in dimerization.

We have considered the possibility that the effect of Tax at very low CREB concentrations could be attributable to a nonspecific carrier effect, in addition to or as an alternative to a direct effect on CREB dimerization. Although it is difficult to exclude this possibility altogether, we found that the effect of Tax could not be mimicked by bovine serum albumin (M.G.A. and W.S.D., unpublished results). Moreover, the effect of Tax is dependent on specific DNA sequences outside the CREB site. One would not expect this to be the case if Tax were acting simply as a nonspecific carrier protein.

Role of CREB phosphorylation

CREB is phosphorylated at serine 119 *in vivo* and *in vitro* by protein kinase A in response to cyclic AMP. The CREB DNA binding domain is remote from the phosphorylation site in the primary sequence, and there is disagreement over the extent to which phosphorylation influences DNA binding (38, 41).

We found that phosphorylation did not affect binding to the CREB site in the HTLV-I TxRE used in our experiments. Moreover, phosphorylated and nonphosphorylated CREB were equally susceptible to the effects of Tax. This suggests that Tax and protein kinase A phosphorylation have different and independent effect on CREB conformation. Our findings are consistent with a previous study showing that Tax and cyclic

AMP had independent effects on HTLV-I gene expression *in vivo* (42).

Participation of Tax in CREB-DNA complexes

It is implicit in our equilibrium binding model that Tax associates with one or more species in the CREB-DNA binding reactions, thus changing the apparent binding parameters. Direct evidence for the association of Tax with DNA bound forms of CREB is provided by the small decrease in electrophoretic mobility seen in most experiments (Figure 1; see also references, (20, 22, 40)). In our experience, the retardation in mobility has been somewhat variable, consistent with a weak interaction, near the limits of what can be detected using the electrophoretic assay. For this reason we have not attempted to interpret the retardation quantitatively.

Certain anti-Tax antibodies prevent CREB-DNA complexes from entering native gels (22, 40). These data strongly support the idea that Tax associates with such complexes, although interpretation is subject to the qualification that the antibody itself could perturb the reaction. A rigorous understanding of the association of Tax with the different species in the CREB-DNA binding reactions will probably require non gel-based assays, which are beyond the scope of the present study.

Despite the difficulty in directly visualizing the presence of Tax in the $(\text{CREB})_2$ -DNA complexes, it is possible to put forward an overall working model consistent with the results reported here and obtained by other laboratories. We suggest that Tax binds CREB and induces a conformational change that favors dimerization, while simultaneously altering the contacts that CREB makes with its DNA binding site. The change may be quite subtle, perhaps merely changing the angle at which CREB passes through the major groove of the DNA so as to convert nonspecific contacts with flanking sequences to specific ones. It is not clear whether Tax directly contacts the DNA in the complex, although the failure to obtain significant UV-crosslinked adducts between Tax and DNA suggests that it may not (M.G.A. and W.S.D., unpublished results, and (26)).

Several aspects of this model remain to be clarified. We do not know the stoichiometry of Tax binding, and we lack direct evidence for the position of Tax in the complex. In addition, the model is directly applicable only to CREB and related proteins. It remains to be learned how Tax is able to affect the binding of proteins in other structural classes (23).

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