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Protein Kinase A-Dependent Binding of a Nuclear Factor to the 21-Base-Pair Repeat of the Human T-Cell Leukemia Virus Type I Long Terminal Repeat

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The long terminal repeat (LTR) of the human T-cell leukemia virus type I (HTLV-I) contains an imperfect repeat of 21 nucleotides which governs the response to the virus *trans*-activator protein *tax* and to cyclic AMP. In a murine thymocyte cell line defective in the catalytic subunit of protein kinase A, the response of the HTLV-I LTR to cyclic AMP is abolished and the response to *tax* is substantially diminished. This report shows that a factor present in nuclear extracts of wild-type cells binds to the HTLV-I 21-nucleotide sequence and that this binding activity is missing from the extracts of protein kinase A-defective cells. Treatment of nuclear extracts of protein kinase A-defective cells with the bovine protein kinase A catalytic subunit restores the binding activity, whereas treatment of wild-type nuclear extracts with a protein phosphatase destroys the binding activity. The binding factor is referred to as protein kinase A-dependent factor (PKAF). These results indicate that in murine thymocytes the response of the HTLV-I LTR to cyclic AMP depends upon the binding of a phosphorylated protein to the 21-nucleotide repeat sequence and that the response to *tax* is partially dependent upon binding of the phosphorylated protein. The results suggest a model in which the phosphorylation of a transcription factor by protein kinase A regulates HTLV-I gene expression.

The human T cell leukemia virus type I (HTLV-I), a retrovirus, is the etiological agent of adult T cell leukemia and lymphoma, an aggressive tumor of the CD4-positive lineage (25, 47, 48), and is associated with tropical spastic paraparesis and HTLV-I-associated myelopathy (20, 30, 65). In addition to the genes which specify virus structural components, the genome of HTLV-I contains an additional region called X, which is capable of immortalizing primary human lymphocytes in the absence of the other HTLV-I genes (21). The X region specifies at least two functional proteins, the *trans*-activator (*tax*), which acts to accelerate the transcription of viral RNA (5, 15, 16, 60), and the regulator of virion protein expression (*rex*), which is required for the accumulation of full-length viral RNA (24, 27, 28). Expression of the X region proteins as well as virus structural genes is controlled by the long terminal repeat (LTR) sequences of the virus which flank the viral genetic information. An understanding of transcriptional control of expression of virus structural and immortalizing genes is essential to an understanding of the life cycle of and tumor induction by the virus.

The HTLV-I LTR is responsive to at least two signals that act in *trans*. The rate of transcription directed by the HTLV-I LTR is increased almost 100-fold by the *tax* protein (5, 15, 16, 60), and 3- to 8-fold by increased levels of cyclic AMP (31, 49). The effect of these two stimuli are more than additive, as the level of LTR activity is increased 200- to 300-fold in the presence of both *tax* and cyclic AMP (49). The sequences within the LTR which are responsive to *tax* and to cyclic AMP overlap one another (18, 31, 49). The 5-nucleotide sequence TGACG, which is required for the response to cyclic AMP and to the *tax* protein, is contained entirely within the 21-nucleotide sequence that is present in three nearly identical copies in the viral LTR (4, 17, 37, 44, 49, 51, 57) (Fig. 1). The supra-additive response of the LTR to both *tax* and cyclic AMP, as well as the overlap of the responsive elements, suggests that cellular transcription factors that promote *tax* activity may be activated by cyclic AMP. In eucaryotic cells, cyclic AMP activates a cellular protein kinase (protein kinase A [PKA]) that modifies the activity of proteins which bind to DNA in the vicinity of promoters (1, 12, 34, 40, 42, 50, 64). The recent observation that the response of the HTLV-I LTR to cyclic AMP was abolished and the response to *tax* was considerably diminished in a murine thymocyte cell line, S49, deficient in PKA (P. K. Kadison, H. T. Poteat, and D. Faller, submitted for publication), prompted a comparison of proteins present in the parental and mutant cell lines which bind to the 21-base-pair (bp) repeat sequence. This report shows that S49 cells contain a factor that binds the 21-bp repeat. The binding activity is absent in the S49 cells deficient in PKA. We also report that the binding activity absent from the PKA-deficient extract can be restored by treatment of the extract with a purified PKA preparation.

MATERIALS AND METHODS

Preparation of end-labeled oligonucleotides. Single-stranded oligonucleotides were synthesized on a DNA synthesizer (model 381A; Applied Biosystems, Inc.). Six single-stranded oligonucleotides 33 bases in length were manufactured. These oligonucleotides had the following sequences: AGCTTCAAGGCGTTGACGACAACCCCTCCC TTT, CTAGAAAGGGAGGGGTTGTCGTCAACGCCT TGA (21HX), AGCTTCAACGGCAGCGGAATCTACCTC TCCTTT, CTAGAAAGGAGAGGTAGATTCCGCTGCCG TTGA (NBHX), and AGCTTCAAGGCGTAAAAAACAAC CCCTCCCTTT, CTAGAAAGGGAGGGGTTGTTTTTTAC GCCTTGA (2BHX). The oligonucleotides were purified by high-pressure liquid chromatography. After heating to 90°C,

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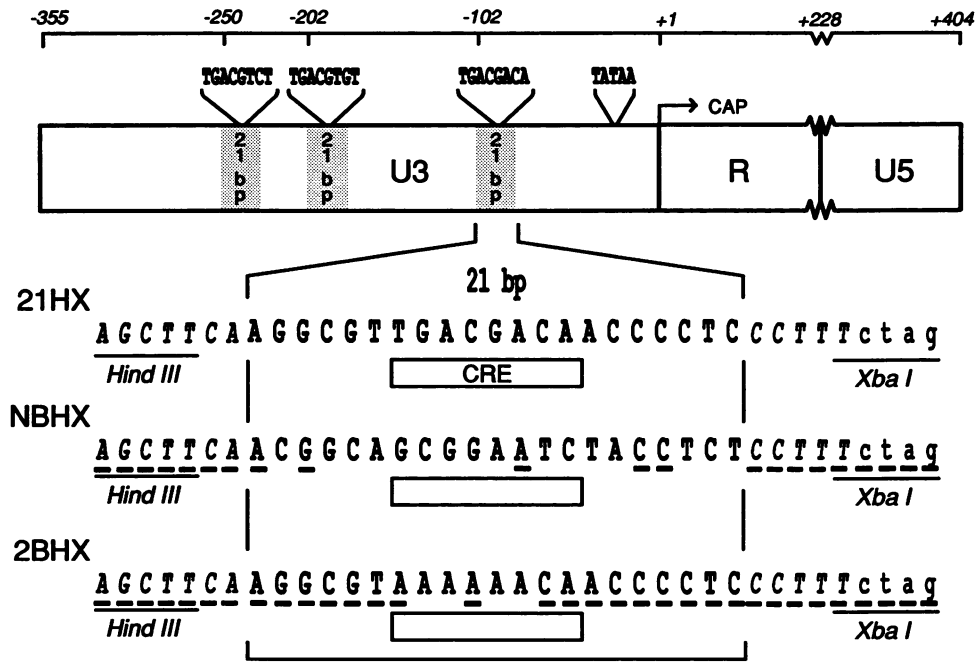

FIG. 1. Schematic diagram of the HTLV-I 5′ proviral LTR. At the top of the diagram a line gives the location of each segment of the LTR: U3, R, and U5. The start site of transcription is labeled +1. The location of the first base pair in each of the 21-bp imperfect repeats is also shown. The cyclic AMP responsive elements (CRE) within each 21-bp repeat are indicated above the block diagram, as are the TATAA box and the start site of transcription. Below the diagram of the LTR, the plus strands of three oligonucleotides are shown. The complementary negative-stranded oligonucleotide was also synthesized but is not shown in the diagram. The viral sequence present in the 21-bp repeat indicated was used in the 21 bp of 21HX. These are in roman capital letters. The flanking sequence is not derived from the virus (italic capital letters). The flanking sequences contain sticky-end overhangs which can be used for end filling or cloning. The CRE of this 21-bp repeat is indicated by a closed box. For the other oligonucleotides, bases which are identical to 21HX are underlined. The boxes below the NBHX and 2BHX sequences indicate the location and sequence of the mutated CRE. The oligonucleotides actually made by the DNA synthesizer are shown in capital letters; the bases that would be expected to arise from end-filling reactions are depicted in lower-case letters.

equal numbers of A_{260} units were annealed in a solution of 100 mM NaCl and 10 mM Tris hydrochloride (pH 7.4). The annealed oligonucleotides were end labeled with Klenow DNA polymerase (41). The probe was purified by using the Gene Clean Kit (model 3501; Bio 101) as specified by the manufacturer. It was checked on a nondenaturing acrylamide gel to assess contamination with single-stranded oligonucleotide. Contamination was estimated at less than 5% and was often not detectable. The average specific activity of an annealed labeled oligonucleotide was 9.0×10^2 dpm/ng of DNA.

Growth of S49 thymocyte cell lines. Wild type S49 24.3.2, designated wild type in the text, and S49 kin$^-$ 24.6.1, designated kinase deficient in the text, were obtained from the University of California San Francisco Tissue Culture Facility. The cells were maintained as described by Wetters et al. (62). Successful growth of the cells also required inspection of cell density every 12 to 16 h, with the cell density maintained between 3.0×10^5 and 9.0×10^5 cells per ml.

Preparation of nuclear extracts. Nuclear extracts were prepared as described by Dignam et al. (10) with modifications. A type A pestle was substituted for a type B pestle, and 20 to 40 strokes of the homogenizer were required for cell or nuclear lysis. From 8×10^8 to 16×10^8 cells which were 100% viable as measured by trypan blue exclusion were harvested by centrifugation at $110 \times g$ for 10 min, washed with phosphate-buffered saline, and centrifuged at $110 \times g$ for 10 min. Any preparation which showed more than 10% nuclear lysis before the appropriate step was discarded. Protein concentration was determined by using the Bio-Rad model 500-0006 protein determination kit as specified by the manufacturer.

Binding of oligonucleotides to factors present in the nuclear extracts. Binding reactions took place in the binding buffer described by Sen et al. (55). It contained 100 mM Tris hydrochloride (pH 7.4), 40% glycerol, 1 mM β-mercaptoethanol, 10 mM EDTA, and 40 mM NaCl. In addition, binding reactions contained 0.2 μg of deoxyinosine and deoxycytosine per μl, alternating copolymer (pharmacia 27-7880-03), 0.05 to 0.25 μg of the indicated nuclear extract, and 2,000 cpm of the indicated oligonucleotide per μl. In all binding reactions except those to which the kinase buffer was added, 2 mM $MgCl_2$ was also present. For binding reactions containing kinase buffer the divalent cation concentration was 2 mM Mg^{2+} and 1 mM Mn^{2+}. Binding reactions were carried out at 4°C for 10 min. The reaction product was then loaded onto a 4% acrylamide 0.5× TBE gel, and electrophoresis was carried out at 150 V for 2 to 4 h at room temperature. Gels were vacuum dried at 65°C for 1 h.

Kinase and phosphatase treatment of nuclear extracts. Kinase reactions were performed as described by Cherry et al. (6). The temperature, time for which the reaction was allowed to proceed, ATP concentrations, and kinase concentration varied as indicated in the figure legends. PKA catalytic subunit (CS) was obtained from Sigma Chemical Co. (no. P-2645) and is the same preparation described by Cherry et al. (6). A 1- to 10-μg quantity of the indicated nuclear extract was used in a final reaction volume of 24 μl. Half of this reaction mixture was added to a 20-μl (final volume) binding reaction.

Phosphatase reactions were performed with a 24-μl reaction volume, with 1 to 10 μg of protein from the indicated nuclear extract. The time and temperature of incubation are indicated in the figure legends. Between 2×10^{-3} and 2 U of calf intestinal alkaline phosphatase (pharmacia 1097-075) con-

tained in an initial volume of 2 μl was used for each reaction. Two units of phosphatase represented 0.64 μg of total protein. To reactions containing less than 2 U of phosphatase, an appropriate amount of bovine serum albumin (BSA) was added to maintain a constant concentration of protein. The buffer for the reaction was that described by Maniatis et al. (41), this CIP buffer contained 50 mM Tris hydrochloride, 1 mM $MgCl_2$, 0.1 mM $ZnCl_2$, and 1 mM spermadine.

RESULTS

The ability of proteins present in the nuclear extracts of S49 cells from the PKA-deficient mutant, S49 kin$^-$ 24.6.1, to bind to the 21-bp repeat of the HTLV-I LTR was examined. The DNA sequence used was an oligonucleotide 33 bp long which contained the sequence of the 3′-most 21-bp repeat of the HTLV-I LTR (33, 54). This sequence was chosen because it confers responsiveness to the *tax* protein and to cyclic AMP (49, 51). The sequence of this oligonucleotide (21HX) is shown in Fig. 1. As a control, a second 33-bp oligonucleotide (NBHX) was made. This oligonucleotide contained the same restriction sites which flanked 21HX, but the sequence of the 21-bp repeat was replaced with a sequence deficient for nuclear-factor binding (59).

Nuclear extracts were prepared from a murine thymocyte S49 cell line deficient for the production of the PKA catalytic subunit (CS) (7). As a control, nuclear extracts were prepared from the wild-type S49 parent cell line. The S49 cell lines were selected for these studies because they are the only cells of the T lymphocyte lineage available which contain well-characterized mutations in the cyclic AMP pathway. Nuclear extracts were prepared by the method of Dignam et al. (10). The total protein content of each extract was determined, and equal amounts of protein were compared in binding reactions on the 21-bp repeat element. Binding of nuclear factors from the S49 kinase-deficient and S49 wild-type cell lines to the 21-bp repeat sequence was measured by using the gel mobility shift assay. In this assay the labeled oligonucleotide is incubated with the nuclear extract in the presence of a binding buffer. The contents of this binding reaction are then loaded onto a nondenaturing polyacrylamide gel. During electrophoresis, DNA molecules which bind nuclear factors migrate more slowly than those which do not.

Three distinct complexes were observed when S49 wild-type nuclear extracts were incubated with the labeled 21-bp repeat oligonucleotide 21HX. The three complexes are denoted a, b, and c respectively (Fig. 2, lane 2). Only complexes a and c were observed when extracts from the S49 kinase-deficient cells were used. Complex b was not present. To determine the sequence specificity of complex formation, we used unlabeled 21HX and NBHX DNAs as competitors in the reaction. Complex a was competed for equally well by the 21HX and NBHX oligonucleotide (Fig. 2). In contrast, both complex b and complex c were inhibited more readily by the 21HX oligonucleotide (Fig. 2, compare lanes 3 and 4 with lanes 5 and 6 or compare lanes 7 and 8 with lanes 9 and 10). More unlabeled 21HX DNA was required to inhibit complex c than to inhibit complex b (Fig. 2, lanes 6 and 9). When the 2BHX oligonucleotide (Fig. 1) was labeled and used in binding reactions, complex b formation was not observed (data not shown). The same patterns of complex formation were found in a second set of kinase-deficient and wild-type S49 nuclear extracts (data not shown).

To determine whether proteins responsible for the formation of complex b in the wild-type S49 cell line might also be present in the mutant S49 cell line but be deficient in the ability to form a complex with the 21-bp repeat DNA, we

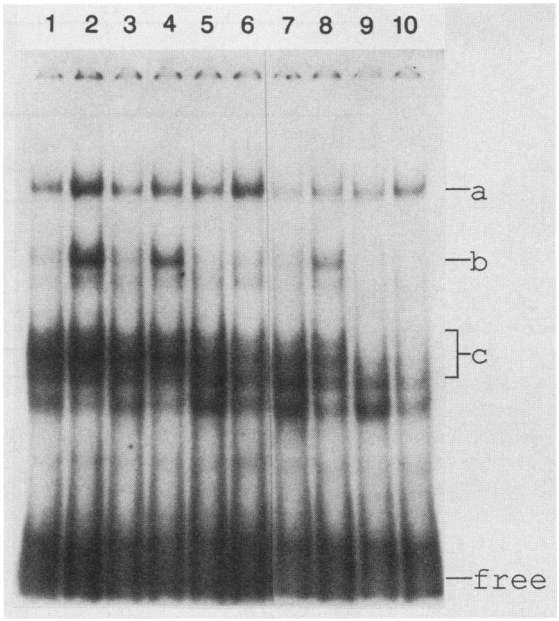

FIG. 2. Gel shift assay showing binding of nuclear extracts from S49 wild-type and S49 kinase-deficient cell lines to the 21-bp enhancer element of the HTLV-1 LTR. Odd-numbered lanes show binding with the S49-kinase deficient extracts; even-numbered lanes show binding with the S49 wild-type extracts. Unlabeled competitor DNA oligonucleotides have been added to some of the binding reactions. The amounts and types of unlabeled cold competitor are as follows: lanes 1 and 2, no competitor added; lanes 3 and 4, 1.92×10^{-3} A_{260} units of NBHX oligonucleotide; lanes 5 and 6, 1.92×10^{-3} A_{260} units of 21HX; lanes 7 and 8, 9.6×10^{-3} A_{260} units of NBHX; lanes 9 and 10, 9.6×10^{-3} A_{260} units of 21HX. The ratio of unlabeled competitor DNA to labeled DNA in each lane was estimated to be 7.2 ± 2.4 for lanes 3 to 6 and 36 ± 12 for lanes 7 to 12. The results shown are from a photograph of a single autoradiogram.

attempted to reconstitute the binding activity by using purified PKA CS which is absent in the S49 kinase-deficient cells. Nuclear extracts were incubated with the CS of bovine PKA and ATP. A portion of this reaction mixture was then added to a binding reaction. The samples were loaded onto a nondenaturing acrylamide gel. This procedure yielded a complex which comigrated with complex b (Fig. 3). When either the wild-type or kinase-deficient extract was incubated in the kinase buffer alone, a decrease in complex formation was noted (Fig. 3A, compare lanes 1 and 2 with lanes 3 and 4). Incubation of the kinase-deficient extracts with PKA CS gave a level of complex b formation greater than or equal to that observed for the wild-type extracts (Fig. 3A, compare lane 3 with lanes 7 to 10). A high level of complex b formation by the kinase-deficient S49 nuclear extracts was dependent on the addition of both ATP and kinase to the kinase reaction. The omission of either ATP (Fig. 3A, lane 5) or kinase (Fig. 3A, lane 6) resulted in a decreased or absent complex b. The conditions specific for phosphorylation of a purified protein at PKA consensus phosphorylation sites have been established (6). When these conditions were duplicated by using the nuclear extract from the kinase-deficient S49 cells, complex b was detected (Fig. 3B, lane 3). These results suggest that incubation of the kinase-deficient nuclear extracts with PKA CS and ATP can result in a level of complex b formation greater than that observed in wild-type extracts and that a detectable level of complex b formation occurs at concentrations of kinase that yield specific phosphorylation of PKA consensus phosphorylation sites.

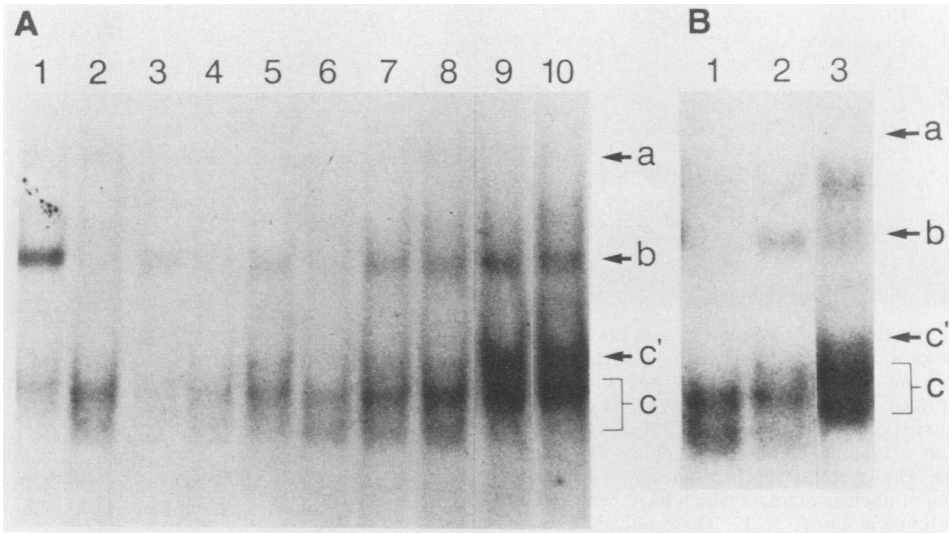

FIG. 3. Comparison of nuclear extracts prepared from S49 kinase-deficient cells for binding to the 21-bp enhancer element after incubation with the PKA CS. (A) Dependence of complex b formation on ATP and kinase. In lanes 1 and 3, S49 wild-type extracts were used for binding reactions; in all other lanes, S49 kinase-deficient extracts were used. Lanes 1 and 2 contain binding reactions without the addition of kinase buffer or preincubation. All other lanes show the result of binding reactions after the addition of kinase buffer and following a preincubation step. Before addition to a binding reaction containing labeled probe and binding buffer, extracts were incubated in buffer only (lanes 3 and 4), buffer plus 1.5 picomolar units (U) of PKA-CS per μl (lane 5), buffer plus 100 μM ATP (lane 6), buffer plus 1.5 U of PKA CS per μl and 40 nM ATP (lane 7), buffer plus 1.5 U of PKA CS per μl plus 100 μM ATP (lane 8), buffer plus 4.7 U of PKA CS per μl and 40 nM ATP (lane 9), or buffer plus 4.7 U of PKA CS per μl and 100 μM ATP (lane 10). Four lanes, which showed complex b formation to be independent of two- to threefold alterations in the amount of nuclear extract added, have been removed from a photograph of the autoradiogram. (B) Specificity of the kinase reaction. Lanes: 1, kinase-deficient extract with no preincubation; 2, wild-type extract with no preincubation; 3, result of preincubation of the S49 kinase-deficient nuclear extracts with kinase buffer and 40 nM ATP at 20°C for 10 min with 6.2×10^{-3} U of PKA CS per μl. A single autoradiogram was photographed to obtain each panel of the figure shown.

The wild-type S49 extracts were treated with 23 and 0.023 U of PKA CS. No new complexes were observed (Fig. 4A, lanes 1 and 2, respectively). The complexes formed as a result of kinase treatment of the kinase-deficient extracts were tested for sensitivity to sequence-specific competition. The results of this experiment are shown in Fig. 4. Competition with low levels of unlabeled 21HX or NBHX oligonucleotide resulted in no detectable decrease in complex formation (Fig. 4A, compare lane 3 with lanes 5 and 9). Competition with 21HX oligonucleotide eliminated complex formation, whereas competition with a similar amount of the NBHX oligonucleotide did not (Fig. 4A, compare lane 6 with lane 10). The amount of unlabeled 21HX oligonucleotide necessary to compete for the products of a kinase reaction was similar to the amount of 21HX oligonucleotide required to compete for complex b in the wild-type extracts. As in the experiment whose results are shown in Fig. 2, more unlabeled 21HX DNA was required to inhibit complex c than complex b (Fig. 4A, compare lane 6 with lane 7). The results of these two sets of experiments suggest that the binding affinity, sequence specificity, and electrophoretic mobility of the complex formed in the kinase-deficient S49 nuclear extracts after treatment with PKA and ATP are the same as those of complex b formed in the wild-type extracts.

The kinase preparation itself was assayed for binding activity. Some preparations of the kinase showed binding activity (Fig. 4B, lane 3). A complex with a similar migration pattern was seen in other binding experiments (Fig. 3A, complex c', lanes 9 and 10). This complex was not dependent on ATP or the time and temperature of incubation and did not comigrate with complex b (Fig. 4B) (data not shown). At lower concentrations of kinase, no binding of the kinase preparation could be detected, nor could complex c' be observed when these same concentrations of kinase were used to elicit complex b formation (Fig. 3A, lanes 7 and 8). These results suggest that complex b does not result from a binding activity present in the kinase preparation.

Effect of phosphatase treatment. The experiments presented above suggested that phosphorylation was important for complex formation. To test this hypothesis, nuclear extracts from both S49 wild-type and S49 kinase-deficient cell lines were incubated with calf intestinal alkaline phosphatase (CIP). Reactions were carried out at 4, 20, and 37°C in the presence or absence of phosphatase buffer. A sample of each phosphatase reaction mixture was then added to a binding reaction.

The phosphatase preparation alone showed no binding activity (Fig. 5A, lane 2). The CIP buffer alone enhanced the complex formation of each factor (Fig. 5A, compare lane 1 with lane 5). Addition of the protein phosphatase to the nuclear extract of wild-type cells dramatically altered both the level and type of complex formation (Fig. 5A). No band which comigrated with complex b could be observed. This new binding pattern appeared when the reaction was carried out at 20 or 37°C (Fig. 5A, compare lane 4 with lane 6). However, when the phosphatase was added at 4°C and no preincubation was allowed to occur, complex formation was not disrupted (data not shown).

As an additional control, phosphatase and nuclear extracts from the S49 kinase-deficient cells were incubated and assayed for binding activity. The results of this experiment are shown in Fig. 5B. A low level of complex b formation was seen when extracts from the kinase-deficient cells were incubated with the CIP buffer (Fig. 5B, lanes 1 and 3). Incubation of the S49 kinase-deficient extracts with 2 U of the phosphatase, but not with smaller amounts, resulted in an altered set of complexes (Fig. 5B, compare lane 1 with lane 2). These new complexes were nearly identical to those

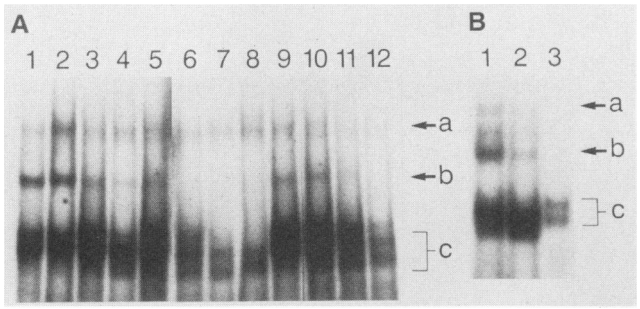

FIG. 4. (A) Kinase treatment of nuclear extracts derived from wild-type S49 cells (lanes 1 and 2). These nuclear extracts were preincubated with 1.5 U of PKA CS per μl and 40 nM ATP (lane 1) or 9.0×10^{-3} U of PKA CS per μl and 40 nM ATP (lane 2). The preincubation was carried out for 10 min at 20°C. A sample of this mixture was added to a binding reaction containing the labeled 21HX oligonucleotide. The effect of specific and nonspecific competition on the binding of nuclear extracts which have been treated with the PKA CS is shown in lanes 3 to 12. These nuclear extracts from kinase-deficient S49 cells were preincubated with various amounts of PKA CS and 40 nM ATP and then added to binding reactions containing the labeled 21HX oligonucleotide. Some of the binding reactions also contained unlabeled competitor oligonucleotides. Lane 3 contains 1.5 U of PKA CS per μl and no competitor oligonucleotide. Lane 4 contains 9.0×10^{-3} U of PKA CS per μl and no competitor oligonucleotide. All subsequent reactions were preincubated with 1.5 U of PKA CS per μl with the following amounts of unlabeled competitor oligonucleotide present in the binding reaction: 2.0×10^{-4} A_{260} units of 21HX (lane 5), 4.8×10^{-3} A_{260} units of 21HX (lane 6), 2.4×10^{-2} A_{260} units of 21HX (lane 7), 0.12 A_{260} units of 21HX (lane 8), 2.0×10^{-4} A_{260} units of NBHX (lane 9), 4.8×10^{-3} A_{260} units of NBHX (lane 10), 2.4×10^{-2} A_{260} units of NBHX (lane 11), and 0.12 A_{260} units of NBHX (lane 12). The ratio of unlabeled competitor DNA to labeled DNA in each lane was estimated to be 0.72 ± 0.24 (lanes 5 and 9), 18 ± 6 (lanes 6 and 10), 90 ± 30 (lanes 7 and 11), and 450 ± 150 (lanes 8 and 12). A lane showing the effect of 9.1×10^{-4} A_{260} units of the NBHX competitor and a blank lane have been removed from a photograph of the autoradiogram. (B) The nuclear extracts used for binding reactions to the end-labeled 21HX oligonucleotide were S49 wild-type cells (lane 1) and S49 kinase-deficient cells (lane 2). No nuclear extract was added to lane 3; only 4.7 U of PKA CS per μl was added to the binding reaction. All extracts were preincubated in kinase buffer. A photograph of a single autoradiogram was used to prepare the figure.

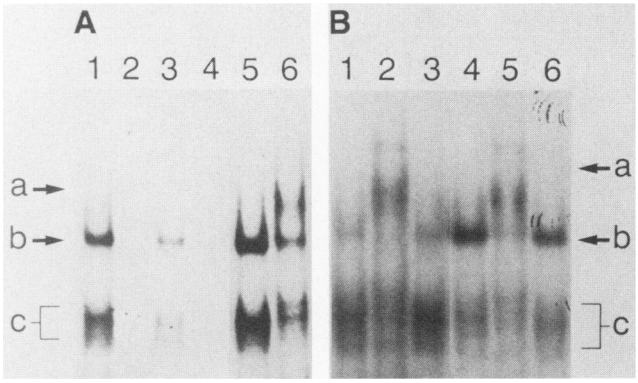

FIG. 5. Effect of CIP on the binding of nuclear factors from S49 wild-type and S49 kinase-deficient cell lines to the 21HX oligonucleotide. (A) Phosphatase reactions on S49 wild-type extracts, showing the effect of phosphatase and CIP buffer on complex formation. In all lanes except lane 2, S49 wild-type nuclear extracts were used in the binding reactions. For lane 2, 2 U of CIP in 1× CIP buffer was added to a binding reaction. Lane 1 contains S49 wild-type extract added directly to the binding reaction without preincubation. For lanes 3 to 6, a 10-min preincubation of the extract was performed. Lanes 3 and 4 were incubated in the absence of 1× CIP buffer at 37°C. In lane 3, no CIP was added; in lane 4, 2.0 U of CIP was used. Lanes 5 and 6 were incubated in the presence of 1× CIP buffer at 20°C. CIP (2 U) was added to lane 6; no CIP was used in lane 5. Panel A was prepared from a photograph of a single autoradiogram. (B) Phosphatase treatment of kinase-deficient and wild-type S49 nuclear extracts. In lanes 1 to 3, nuclear extracts from S49 kinase-deficient cells were used for binding reactions; in lanes 4 to 6, nuclear extracts from wild-type S49 cells were used. The extracts were incubated for 10 min at 20°C in 1× CIP buffer with the following amounts of CIP enzyme and BSA: lanes 1 and 4, 0.2 U of CIP (60 ng of protein) and 540 ng of BSA; lanes 2 and 5, 2.0 U of CIP (600 ng of protein); lanes 3 and 6, no CIP or BSA added. After preincubation, portion of the phosphatase reaction mixture was added to a binding reaction, which contained labeled 21HX oligonucleotide. Panel B was prepared from a photograph of a single autoradiogram.

formed when an equal amount of phosphatase was incubated with the wild-type S49 extract (Fig. 5B, compare lane 2 with lane 5). Therefore, after a dephosphorylation reaction, the kinase-deficient extracts and the wild-type extracts form nearly identical complexes.

DISCUSSION

The experiments described here demonstrate that nuclear extracts of murine T lymphocytes contain a protein which binds to the 21-nucleotide repeat sequence of the HTLV-I enhancer and that the activity of this factor is greatly diminished in extracts of cells defective for PKA CS. The binding activity appears to be dependent upon phosphorylation, since it can be restored by treatment of nuclear extracts of PKA-deficient cells with a protein kinase or inhibited by treatment of the wild-type extracts with the protein phosphatase. We propose to call this binding activity the PKA-dependent binding factor (PKAF). PKAF may be an essential component of the response of HTLV-I LTR to cyclic AMP in murine thymocytes. No stimulation of the HTLV-I LTR by cyclic AMP is observed in cells deficient for this binding activity. PKAF may also contribute to the response of HTLV-I to *tax* in these cells, since the response of the viral LTR to *tax* is diminished in PKA-deficient cells (Kadison et al., submitted). However, it is also possible that the PKA deficiency prevents the alteration of proteins other than PKAF important for *tax* activity, even though the *tax* and cyclic AMP responsive sequences overlap. These data suggest a role for PKA-mediated phosphorylation in the control of HTLV-I gene expression.

A second sequence-specific complex, complex c, was present in mutant and wild-type cell lines. For the purpose of discussion this factor is called PKA-independent factor (KAIF). PKA treatment of the extracts increased the level of PKAF and KAIF binding (Fig. 3). Phosphatase treatment decreased the binding of PKAF and KAIF. The result of the phosphatase experiment suggests that phosphorylation may be a general mechanism regulating binding to the 21-bp repeat. Chromatographic fractionation of the nuclear extracts suggest that KAIF has a molecular mass of 45 to 65 kilodaltons. Chromatographic fractionation resulted in a loss of PKAF-binding activity (data not shown). Binding reactions carried out with the 2BHX oligonucleotide (Fig. 1) suggest that KAIF complex formation does not involve the cyclic AMP responsive element, TGACG. It is therefore unlikely that the PKAF-binding activity (complex b) derives from dimerization of KAIF (complex c). This is significant because the binding of dimers but not of monomers of the cyclic AMP responsive element-binding protein described by Yamamoto et al., CREB, is dependent on phosphoryla-

tion (64). The data presented here suggest that PKAF binding is regulated in a manner related to but distinct from regulation of the CREB protein.

The effects of cyclic AMP on T cells are, in general, antiproliferative. Both interleukin-2 production and T-cell cytotoxicity are decreased by cyclic AMP (3, 9, 14, 23, 29, 32, 34, 38, 46). The effect of cyclic AMP on T cells infected with HTLV-I may be different. Cyclic AMP may increase the levels of viral regulatory and structural proteins which cause lymphocyte growth (8, 11, 19, 21, 26). Activation of PKA by cyclic AMP may be an important mechanism for inducing HTLV-I gene expression from a transcriptionally silent provirus. The HTLV-II LTR, bovine leukemia virus LTR, and several early promoters of adenovirus have enhancers which respond to viral *trans*-acting factors and cyclic AMP (13, 22, 35, 53, 56). The result that PKA regulates the binding of a nuclear factor to the HTLV-I LTR may allow a better understanding of how other oncogenic viruses control gene expression.

The best-described *tax*-responsive enhancers are the 21-bp repeat of the HTLV-I LTR and the kB enhancer present in the interleukin-2 receptor α-chain promoter and in the human immunodeficiency virus type 1 LTR (2, 8, 36, 39, 52). Nuclear factor kB or a related factor binds the kB enhancer in response to PKC (58). The 21-bp repeat and the kB enhancer share neither sequence similarity nor a similar level of basal factor binding (31, 43, 45, 61). Even so, transcriptional activation of each enhancer can be accomplished by *tax* or by phosphorylation of a DNA-binding factor (58, 63). The specificity of *tax* for certain cellular and viral enhancers may be correlated with the inducibility of these enhancers by phosphorylated transcriptional factors.

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