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The Rex Proteins of Human T-Cell Leukemia Virus Type II Differ by Serine Phosphorylation

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The Rex proteins of human T-cell leukemia virus types I and II (HTLV-I and HTLV-II) induce cytoplasmic expression of unspliced *gag-pol* mRNA and singly spliced *env* mRNA and are critical for virus replication. Two *rex* gene products, $p27^{rex}$ and $p21^{rex}$ of HTLV-I and $p26^{rex}$ and $p24^{rex}$ of HTLV-II, have been detected in HTLV-infected cells; however, the structural and biological relationship of the proteins has not been clearly elucidated. Endoproteinase digestion and phosphoamino acid analysis of HTLV-II Rex indicated that $p24^{rex}$ has the same amino acid backbone as $p26^{rex}$ and that the larger apparent molecular size of $p26^{rex}$ is attributable to serine phosphorylation.

The human T-cell leukemia virus type II (HTLV-II) *rex* gene encodes two proteins of 26 and 24 kDa (10, 11). Analogous *rex* gene products of 27 and 21 kDa are also encoded by HTLV-I (7, 8). The structural and biological relationship of the two *rex* gene products has not been clearly defined, although it has been established that both proteins share the same carboxy terminus (10, 11). Mutations that remove the first methionine (Met) initiation codon of the *rex* open reading frame result in loss of Rex regulatory function. One study provided indirect evidence through in vitro translation, suggesting that HTLV-I $p21^{rex}$ results from initiation at an internal AUG of the *tax-rex* mRNA (8). For HTLV-II, our previous studies indicated that $p24^{rex}$ was not expressed if the Met for $p26^{rex}$ was deleted, suggesting a more complex relationship. We recently provided direct evidence that HTLV-II $p24^{rex}$ does not initiate at an internal methionine codon (6). In this report, we further investigate the structural relationship of the two *rex* gene products of HTLV-II, and our results demonstrate that posttranslational modification of $p24^{rex}$ by phosphorylation is responsible for conversion to $p26^{rex}$.

Detection of Rex protein in transfected cells. The protein encoded by the HTLV-II *rex* gene open reading frame consists of 170 amino acids and has a molecular mass of 19 kDa (Fig. 1A). Previous studies have indicated that immunoprecipitation of HTLV-II-infected cell lysates with Rex-specific antisera, followed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), resulted in detection of two proteins, $p26^{rex}$ and $p24^{rex}$ (10, 11). In these studies, HTLV-II wild-type and mutant *rex* were expressed from the *tax-rex* cDNA expression vector BC20.2 (Fig. 1B). Constructs were transfected into the simian virus 40-transformed monkey kidney cell line COS by electroporation (2), and the synthesis of Rex was verified by radioimmunoprecipitation and SDS-PAGE as previously described (6). Both $p26^{rex}$ and $p24^{rex}$ were detected in BC20.2-transfected cell lysates (Fig. 2). Previous characterization of complete HTLV-II proviruses expressing the *rex* internal Met codon point mutations M1, M2, and M3 (see Fig. 1B for location) indicated that these internal Met codons were not necessary for $p24^{rex}$ production and were not crucial to the function of Rex and the transforming properties of the virus (6). Characterization of these point mutations in the BC20.2 expression vector (Fig. 1B) indicated that they produced $p26^{rex}$ and $p24^{rex}$ at levels comparable to wild-type levels (Fig. 2), thus confirming our previous results that these point mutations affect neither Rex activity nor expression of the smaller *rex* gene product (6). The construct BC20.2ΔAcc-Cla, which contains a 126-bp in-frame deletion (*Acc*I to *Cla*I) in both *tax* and *rex* coding sequences (Fig. 1B) expressed two Rex proteins with approximate sizes consistent with a 42-amino-acid deletion, which we term $p23^{\Delta rex}$ and $p21^{\Delta rex}$ (Fig. 2 and 4A). The ability to detect two *rex* gene products in cells transfected with BC20.2ΔAcc-Cla, with sizes consistent with the constructed deletion, demonstrates that both $p26^{rex}$ and $p24^{rex}$ contain these deleted amino acids. The *rex* deletion in BC20.2ΔAcc-Cla is upstream of Met-80, which is mutated in M3, and removes Met-63, which is mutated in M2, thus providing further evidence that $p24^{rex}$ is not an internally initiated product.

Endoproteinase Glu-C digestion of Rex. Since $p26^{rex}$ and $p24^{rex}$ are both immunoprecipitated with antisera directed against the carboxy terminus of Rex and are apparently not independent translation products, the difference in the apparent size of the proteins may be due to posttranslational modification involving glycosylation, phosphorylation, or peptide cleavage. Glycosylation is most likely not responsible for the size difference, since studies have indicated that HTLV-I $p27^{rex}$ and $p21^{rex}$ are not glycoproteins (7). To determine whether the difference in size was due to peptide cleavage or phosphorylation and which region of the protein accounted for this difference in size, we performed endoproteinase digestion on the wild-type Rex proteins as well as the internally deleted Rex proteins.

Metabolic labeling of Rex with [^{35}S]methionine and [^{35}S]cysteine results in the majority of the ^{35}S incorporation taking place in the amino half of the protein, since there is a total of five methionines and cysteines in the first 80 amino acids and only two in the remainder of the protein (Fig. 1A). Endoproteinase Glu-C was chosen for our peptide cleavage analysis, since Rex contains only four endonuclease Glu-C cleavage sites (at Glu-102, Glu-130, Glu-136, and Glu-168 [Fig. 1A]) that would give rise to a major protein digestion

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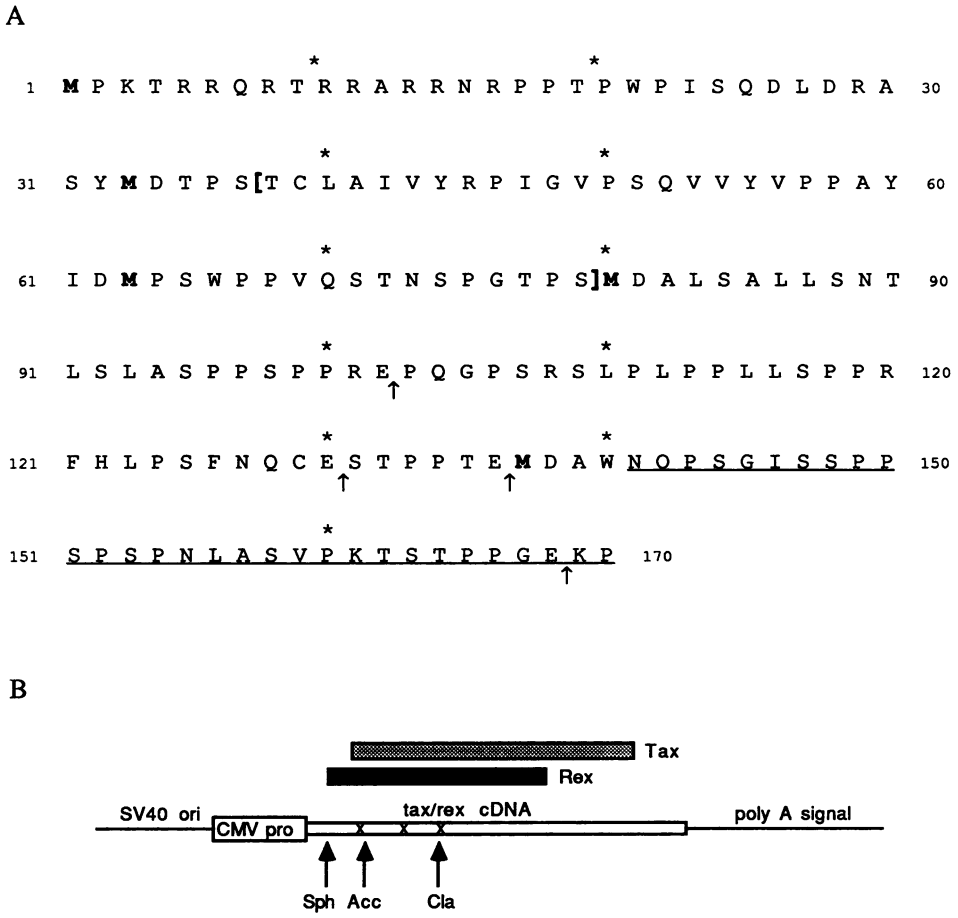

FIG. 1. (A) The complete 170-amino-acid sequence of the Rex protein, based on the nucleotide sequence of the proviral clone pH6neo, is presented (3). The locations of the methionines (M) are depicted in boldface type; the locations of the endoproteinase Glu-C (E) cleavage sites are indicated by arrows. The amino acids deleted in the Rex protein expressed from BC20.2ΔAcc-Cla are bracketed. The underlined region at the carboxy terminus depicts the peptide against which the Rex-specific antisera were directed in this study. (B) The expression vector BC20.2, a BC12-derived vector (5) containing the HTLV-II *tax-rex* cDNA inserted downstream of the cytomegalovirus promoter (CMV pro), is depicted. Shaded boxes represent the *rex* and *tax* open reading frames. Locations of the M1, M2, and M3 internal *rex* gene point mutations, which, upon translation, substitute a threonine for an internal methionine, are indicated (x) and have been previously described (6). Location of the simian virus 40 origin of replication, poly(A) addition signal, and pertinent restriction sites (*Sph*I, *Acc*I, and *Cla*I) are indicated. The drawing is not to scale.

product containing the five methionines and cysteines from the amino portion of the protein. COS cells transfected with BC20.2 or BC20.2ΔAcc-Cla were metabolically labeled with [^{35}S]methionine and [^{35}S]cysteine, and cell lysates were subjected to immunoprecipitation and SDS-PAGE. Wild-type and mutant Rex proteins were individually excised from the dried acrylamide gel and partially digested with endoproteinase Glu-C (4, 9). The digested products were visualized after electrophoresis and autoradiography. Digestion of p26^{rex} and p24^{rex} resulted in detection of the same two major partial digestion products (Fig. 3). From these results, we conclude that the identical peptides are present in both proteins. We confirmed that the major endoproteinase Glu-C digestion products would represent the amino portion of Rex by digestion of p23$^{\Delta rex}$ and p21$^{\Delta rex}$, which contain a deletion of 42 amino acids in the amino half of Rex. Digestion of both these proteins resulted in detection of the same two partial digestion products (Fig. 3). In this particular experiment, the upper partial digestion product for p21$^{\Delta rex}$ was detected in a small quantity; however, subsequent experiments more clearly resulted in two digestion products (data not shown). The digestion pattern of the wild-type proteins was similar to that of the deleted proteins, with the exception of the smaller size of the products, which is consistent with the deletion. These results indicate that the difference in the apparent sizes of p26^{rex} and p24^{rex} is not due to cleavage of at least the amino-terminal 102 amino acids. It is also highly unlikely that the size difference is the result of cleavage in the carboxy portion of the protein, since the antibody used in these experiments is directed against the carboxy-terminal 30 amino acids of Rex. Therefore, we conclude that the amino acid backbones of p26^{rex} and p24^{rex} are the same.

Phosphorylation of Rex. Phosphorylation of the HTLV-II Rex proteins was examined by metabolic labeling of BC20.2- or BC20.2ΔAcc-Cla-transfected COS cells with $^{32}P_i$ followed by immunoprecipitation and SDS-PAGE. ^{35}S-labeled cell lysates were analyzed in parallel. p26^{rex} was easily detected; however, little if any p24^{rex} could be detected (Fig. 4A). Like the wild type, p23$^{\Delta rex}$ incorporated ^{32}P very efficiently. p21$^{\Delta rex}$ was also detected, but at a level approximately

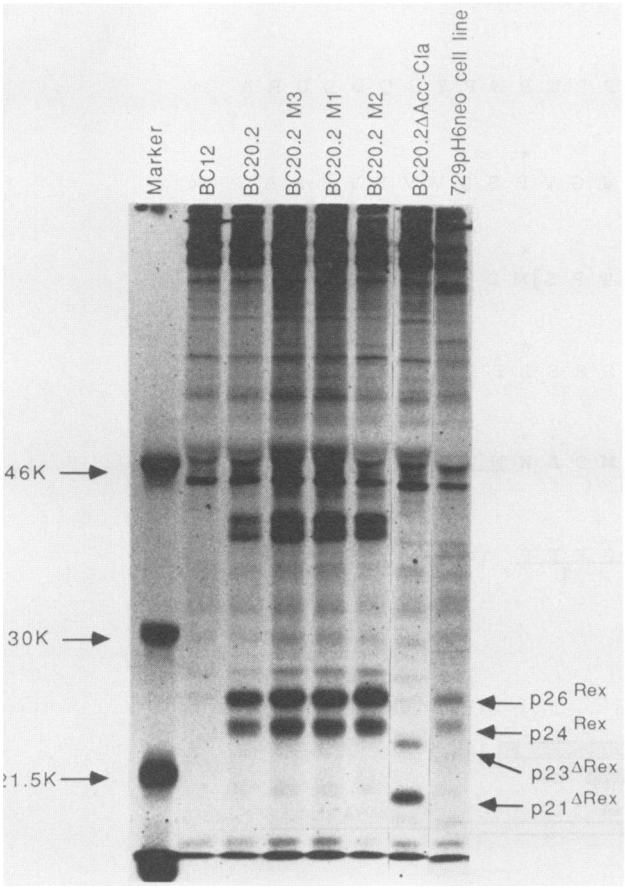

FIG. 2. Detection of Rex in transiently transfected COS cells. COS cells transfected with 10 μg of various expression vector constructs were metabolically labeled with [^{35}S]methionine and [^{35}S]cysteine (Trans ^{35}S-label; specific activity, >1,000 Ci/mmol, >37 TBq/mmol; ICN Biochemicals, Inc.; 100 μCi/ml for 3 h), and cell lysates were prepared as described previously (6). Transfected COS cell lysates, as labeled at the top, were immunoprecipitated with antisera directed against the COOH-terminal tridecapeptide sequence encoded by *rex* (11). The wild-type p26^{rex} and p24^{rex} and the deletion mutants, p23$^{\Delta rex}$ and p21$^{\Delta rex}$, are as labeled on the right. Lysate from the HTLV-II producer cell line 729pH6neo was immunoprecipitated as a positive control. ^{14}C-labeled protein markers (Amersham) are indicated on the left. K, Kilodaltons.

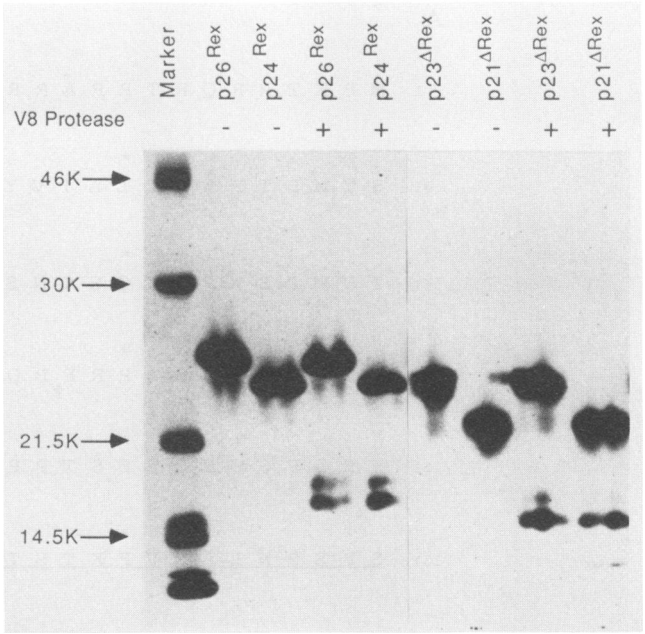

FIG. 3. Partial proteolysis of Rex with endoproteinase Glu-C. ^{35}S-labeled Rex proteins were isolated by immunoprecipitation and SDS-PAGE from lysates of COS cells transfected with BC20.2 or BC20.2ΔAcc-Cla, as described elsewhere (6). p26^{rex}, p24^{rex}, p23$^{\Delta rex}$, and p21$^{\Delta rex}$ were individually excised from the dried gel and partially digested (+) or mock digested (−) during electrophoresis through a 12% polyacrylamide gel with 500 ng of endoproteinase Glu-C (V8 protease isolated from *Staphylococcus aureus*; Boehringer-Mannheim) as described elsewhere (4, 9). ^{14}C-labeled protein markers (Amersham) are indicated on the left. K, Kilodaltons.

fivefold lower than p23$^{\Delta rex}$. Phosphoamino acid analysis (1) of p26^{rex} indicated that p26^{rex} was phosphorylated primarily on serine, with detectable phosphothreonine and phosphotyrosine (Fig. 4B). Phosphoamino acid analysis of HTLV-I p27^{rex} showed similar results (Fig. 4B) and was consistent with previously reported results (7).

Both ^{32}P- and ^{35}S-labeled p26^{rex} were digested with endoproteinase Glu-C and compared on the same gel in an attempt to localize the regions of p26^{rex} which are phosphorylated. The ^{32}P- and ^{35}S-labeled p26^{rex} digestion patterns were identical and were the same as the pattern seen in Fig. 3 for p26^{rex}, suggesting that the majority of phosphorylation occurs within the first 102 amino acids of Rex (data not shown). Our data are consistent with the possibility that the p26^{rex} and p24^{rex} size difference is due to phosphorylation of serine residues.

In vitro dephosphorylation of Rex. To directly test whether the difference in apparent size of p26^{rex} and p24^{rex} is due to phosphorylation, the proteins were treated with bacterial alkaline phosphatase (BAP) in vitro. COS cells were transfected with BC20.2 and metabolically labeled with [^{35}S]methionine and [^{35}S]cysteine, and cell lysates (1 ml) were immunoprecipitated with Rex-specific antisera. The immune complexes were treated with BAP or mock treated and analyzed by SDS-PAGE. Our results show that BAP treatment results in loss of the larger *rex* gene product and an increase in a protein of the same size as p24^{rex} (Fig. 5A). Similar results were seen with Rex expressed from BC20.2ΔAcc-Cla (Fig. 5B). (Note that in this particular experiment, it was not apparent that BAP treatment resulted in an increase in the levels of p21$^{\Delta rex}$, since different amounts of lysate were immunoprecipitated in the BAP- and the mock-treated lanes.) Following BAP treatment, the resulting Rex protein, termed p26^{rex}/p24^{rex}-BAP, was compared with untreated Rex proteins after endoproteinase Glu-C digestion to determine whether BAP treatment altered the mobility of digested peptides. As predicted, the endoproteinase digestion pattern of p26^{rex}/p24^{rex}-BAP was identical to that of p24^{rex} (data not shown).

In conclusion, these results clearly demonstrate that the difference in the apparent size of p26^{rex} and p24^{rex} is the result of phosphorylation. The most likely scenario consistent with our results is that p24^{rex} is the primary Rex translation product and that it is converted to p26^{rex} posttranslationally by phosphorylation on serine residues. The precise location of phosphorylation was not determined, although it appears to be in the first 102 amino acids.

It remains unclear what role, if any, phosphorylation plays

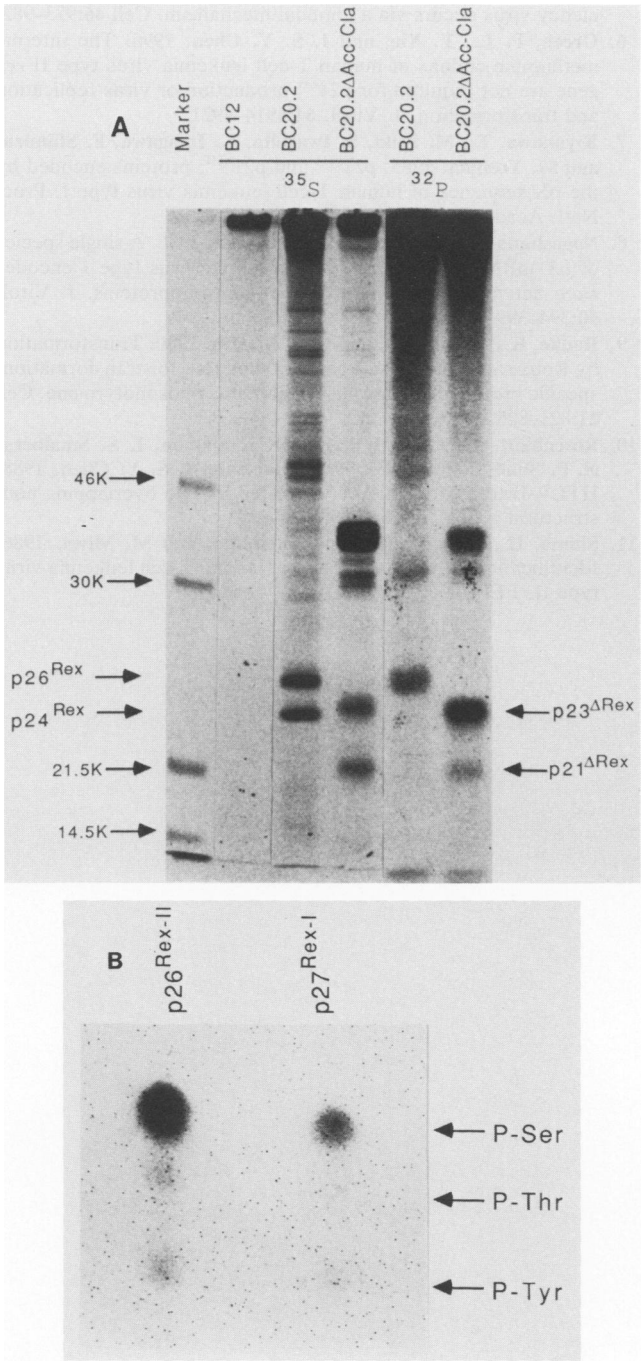

FIG. 4. (A) Detection of Rex in transiently transfected COS cells. COS cells transfected with 10 μg of BC20.2 or BC20.2ΔAcc-Cla were metabolically labeled with either [^{35}S]methionine and [^{35}S]cysteine (as described in the legend to Fig. 2) or $^{32}P_i$ (specific activity, 285 Ci/mg of P, 10.5 TBq/mg of P; ICN Biochemicals; 1 mCi/ml for 3 h), and cell lysates were prepared. ^{35}S- or ^{32}P-transfected cell lysates, as labeled at the top, were immunoprecipitated with Rex-specific antisera. The wild-type p26^{rex} and p24^{rex} and the deleted p23$^{\Delta rex}$ and p21$^{\Delta rex}$ are labeled on the left and right, respectively. We observed that the deleted Rex proteins migrated slightly more slowly in this particular experiment than in our other experiments, which we attribute to acrylamide gel and electrophoresis time variations. ^{14}C-labeled protein markers (Amersham) are indicated on the left. K, Kilodaltons. (B) Identification of the phosphorylated amino acids of p26^{rex-II} (HTLV-II) and p27^{rex-I} (HTLV-I). ^{32}P-labeled p26^{rex-II} and p27^{rex-I} were isolated by immunoprecipitation and SDS-PAGE from lysates of COS cells transfected with BC20.2 and BC1.9 (an HTLV-I *tax-rex* cDNA expression vector). The proteins were eluted from the dried gel, and the precipitated protein was hydrolyzed in 100 μl of 6 N HCl for 2 h at 100°C. Samples were separated on thin-layer cellulose by electrophoresis, and phosphoamino acids were identified by comigration with cold phosphoamino acid standards. The positions of the ninhydrin-positive cold phosphoamino acid markers are indicated on the right.

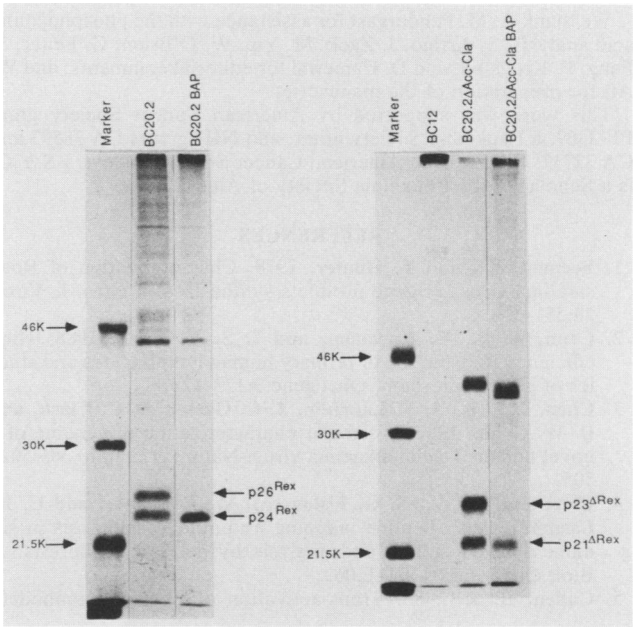

FIG. 5. Dephosphorylation of Rex with BAP. Transfected COS cells were metabolically labeled with [^{35}S]methionine and [^{35}S]cysteine, and cell lysates were prepared as described previously (6). Transfected cell lysates, as indicated at the top, were immunoprecipitated with Rex-specific antisera. The immune complexes were incubated with BAP (150 U for 60 min at 65°C in 50 mM NaCl–10 mM Tris–10 mM $MgCl_2$; total volume, 50 μl) or mock treated, and then they were subjected to SDS-PAGE. The wild-type p26^{rex} and p24^{rex} (left panel) and the deleted p23$^{\Delta rex}$ and p21$^{\Delta rex}$ (right panel) are indicated. The BC20.2ΔAcc-Cla BAP lane contains one-fifth the amount of lysate in the BC20.ΔAcc-Cla (mock-treated) lane. The exposure time for the right panel was 24 h, with the exception of the BAP-treated lane, which was exposed for 72 h. ^{14}C-labeled protein markers (Amersham) are indicated on the left of each panel in kilodaltons (K).

in Rex function. However, phosphorylation has been recognized as a major posttranslational regulatory mechanism and is thought to play an important role in the control of cell growth and differentiation. It is possible that phosphorylation is necessary for activation of Rex function and in addition results in a mobility shift as seen in SDS-PAGE because of charge or conformational changes. We are currently testing whether phosphorylation has a role in the function of Rex. One implication of these results is that HTLV-II gene expression may have additional regulatory controls at the cellular level. A virus such as HTLV that encodes its own regulatory genes would be better able to adapt if it could also respond to regulatory signals of cells it infects. One way in which this could be accomplished would be by having Rex function be dependent on phosphorylation.

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