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Phosphorylation of bovine leukemia virus Tax protein is required for *in vitro* transformation but not for transactivation

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The Tax proteins of the oncovirinae viruses are phosphorylated transcriptional activators that exhibit oncogenic potential. The role of phosphorylation in their functional activities remains unknown. As a model for the Human T-cell leukemia virus type I (HTLV-I), Bovine Leukemia Virus (BLV) permits the characterization of viral replication and leukemogenesis *in vivo*. Here, we show that the BLV Tax protein is phosphorylated on serine residues 106 and 293 both in insect and in mammalian cells. These sites can also be efficiently phosphorylated by the cdc2 and MAP kinases *in vitro*. Mutation of these residues does not affect the capacity of the Tax protein to function as a transactivator. Indeed, the Tax proteins mutated at one or both serines increase LTR-directed viral transcription at levels similar to those obtained with wild-type Tax in cell culture. Moreover, inhibition of Tax phosphorylation by W7, a calmodulin antagonist, does not alter its transactivation activity. Thus, phosphorylation on serines 106 and 293 is not required for transactivation by Tax. However, simultaneous substitution of both serines into alanine residues destroys the capacity of Tax to cooperate with the Ha-*ras* oncogene to transform primary rat embryo fibroblasts and induce tumors in nude mice. When the serines were replaced with aspartic acid residues, the oncogenic potential of Tax was maintained indicating that the negative charge rather than the phosphate group itself was required for Tax oncogenicity. Finally, to assess the role of the serine residues *in vivo*, recombinant viruses which express the Tax mutants were constructed and injected into sheep. It appeared that the mutated proviruses replicate at levels similar to the wild-type virus *in vivo*. We conclude that Tax phosphorylation is dispensable for transactivation and viral replication *in vivo* but is required for its oncogenic potential *in vitro*.

Keywords: ras cooperation; B lymphocyte; sheep; HTLV

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Introduction

Phosphorylation is an essential post-translational modification in eucaryotic cells which governs a series of mechanisms required for the regulation of transcription (reviewed by Hunter and Karin, 1992). Three main levels of regulation modulate transcription factor activity. First, phosphorylation intervenes in translocation of the transcription factor between the cytosol and the nucleus allowing the access to its target sequences. Perhaps the best characterized example of this type of regulation is provided by the Rel-related family of transcription factors. These proteins are sequestered in an inactive form in the cytoplasm and, in response to an exogenous stimulus, are released and translocated to the nucleus where they bind to specific target sequences. This mechanism is illustrated by NF-κB which is retained in an inactive complex in the cytoplasm by the inhibitory protein IκB (Baeuerle and Baltimore, 1988). Phosphorylation of the IκB regulatory subunit triggers nuclear import of NF-κB. The second level where phosphorylation modulates transcription factor activity is DNA binding. One example of negative regulation by phosphorylation is the c-Myb protein. Phosphorylation of c-Myb on serines 11/12 by casein kinase II results in a large decrease in DNA binding activity (Lüscher *et al*., 1990). In contrast, phosphorylation of the serum response factor (SRF) by casein kinase II (CKII) stimulates binding to the c-*fos* promoter (Manak and Prywes, 1991; Janknecht *et al*., 1992; Marais *et al*., 1992). Finally, a third mechanism by which phosphorylation alters transcription is stimulation of transactivation. In this case, phosphorylation can affect the interaction of transactivation domains with the transcriptional machinery. For example, phosphorylation of c-Jun on serines 63/73 in response to mitogenic stimulation is responsible for a concomitant increase of transactivation potential (Smeal *et al*., 1991). It thus appears that phosphorylation can modulate transcription by different mechanisms including protein translocation, DNA binding activities and interactions with the transcriptional machinery.

The Tax proteins of the oncovirinae viruses, a subfamily which includes the Human T cell Leukemia Virus (HTLV-I) and Bovine Leukemia Virus (BLV), have been shown to be phosphorylated (Nyunoya *et al*., 1988; Chen *et al*., 1989; Fontes *et al*, 1993). These proteins are transactivators of viral transcription and modulate the

expression of several cellular genes (reviewed by Kettmann *et al*., 1994 and by Yoshida, 1996). One of the mechanisms by which the Tax proteins activate transcription is dimerisation of the CREB factor (Zhao and Giam, 1992; Perini *et al*., 1995; Baranger *et al*., 1995; Boros *et al*., 1995). Tax itself also acts as a dimer to enhance the DNA binding activity of CREB (Tie *et al*., 1996). Another mechanism of transactivation by Tax involves the NF-κB family of transcription factors. By facilitating the phosphorylation and degradation of IκB and through interactions with IκBα or NFκB precursors, Tax is able to increase the nuclear localization of NFκB (reviewed by Yoshida, 1996). Finally, HTLV-I Tax interacts directly with $p67^{SRF}$ to stimulate the expression of the cellular proto-oncogene c-*fos*. Thus, Tax activates transcription through three different enhancers: the cyclic-AMP responsive element, the NF-κB-binding site and the serum-responsive element. These pleiotropic effects allow to activate transcription from various types of promoters and modulate the expression of several cellular genes (reviewed by Kettmann *et al*., 1994 and by Yoshida, 1996). These functions of HTLV-I Tax appear to be mediated by different domains of the protein (Smith and Greene, 1990; Semmes and Jeang, 1992).

Besides their role in the regulation of transcription, the Tax proteins also exhibit an oncogenic potential (Nerenberg *et al*., 1987; Grassmann *et al*., 1989, Pozzati *et al*., 1990; Tanaka *et al*., 1990; Willems *et al*., 1990; Grossman *et al*., 1995; Akagi *et al*., 1997; Matsumoto *et al*., 1997). Mice transgenic for the *tax*-I gene develop either mesenchymal tumors or leukemia depending on the promoter used for gene expression (Nerenberg *et al*., 1987; Grossman *et al*., 1995). In addition, the Tax protein is able to immortalize both fibroblasts and T lymphocytes in culture in the context of an Herpes saimiri virus-based vector (Grassmann *et al*., 1989; Tanaka *et al*., 1990). The Tax proteins from HTLV-I and BLV behave as immortalizing oncogenes since they are able to cooperate with the Ha-ras oncoprotein to fully transform primary rat embryo fibroblasts (Pozzati *et al*., 1990; Willems *et al*., 1990; Matsumoto *et al*., 1997). Both transactivation and immortalizing functions of Tax can be dissociated by mutations in specific regions of the protein. Indeed, the transactivation of the LTR promoter is not required for Tax to transform these cells (Willems *et al*., 1992; Yamaoka *et al*., 1996). Finally, Tax is an essential gene for viral replication since BLV recombinant viruses that lack transactivation potential are not infectious *in vivo* (Willems *et al*., 1993). It thus appears that the Tax protein plays a key role in the oncogenic potential and in the replication of the virus.

The role of Tax phosphorylation in transactivation and immortalization activities is currently unknown. The aim of this report is to help to gain insight into the role of phosphorylation in immortalization and transactivation by Tax.

Results

Two tryptic peptides from Tax are phosphorylated on serine residues

Bovine leukemia virus is currently the best animal model for HTLV-I because it allows the characterization of the pathogenesis induced by mutant proviruses after direct injection into sheep. It is thus possible to analyse the infectious potential, the viral replication and the leukemogenesis process *in vivo*. This is particularly important because contradictory data arise between *in vitro* and *in vivo* experiments. For example, the deletion of the open reading frames located between the *env* and *tax* genes does not alter the behavior of the BLV and HTLV-II viruses in cell culture but drastically decreases the proviral loads *in vivo* (Willems *et al*., 1994; Green *et al*., 1995). Similar data were obtained with the HTLV-II virus in rabbits but this system does not yet allow to analyse the leukemogenic process *in vivo* (Cockerell *et al*., 1996). Since the BLV model provides a good system for the understanding of HTLV-induced pathogenesis, we chose to characterize the role of Tax phosphorylation in this system.

To gain insight into the role of phosphorylation in either transactivation or immortalization by BLV Tax, we mapped the precise location of the phosphorylation sites. To obtain sufficient amounts of peptides for direct sequencing, the BLV Tax protein was first synthesized in insect SF9 cells using the baculovirus expression system (according to Chen *et al*., 1989). SF9 cells expressing Tax were metabolically labeled with ^{32}P orthophosphate. After purification, ^{32}P-labeled Tax protein was acid-hydrolyzed and analysed by two-dimensional thin-layer chromatography (TLC). Two major and a few minor peptides were revealed after autoradiography (Figure 1a). Since post-translational modifications do not always occur efficiently in insect cells (Luckow and Summers, 1988), phosphorylation of Tax was also analysed in mammalian cells. To this end, Cos cells were transfected with the pSGTax plasmid, an expression vector of the BLV *tax* gene (Willems *et al*., 1990). Twenty-four hours post-transfection, the cells were labeled overnight with ^{32}P orthophosphate. The Tax protein was then immunopurified using a mixture of monoclonal antibodies, digested with trypsin and analysed by TLC. Two labeled peptides co-migrating with those obtained from the insect SF9 cells were observed (Figure 1a). It thus appears that the Tax protein is phosphorylated on two major sites both in insect and in mammalian cells. We next determined the type of residue which was phosphorylated. Therefore, the Tax protein was metabolically labeled with ^{32}P orthophosphate, purified as described above and acid-hydrolyzed in chlorhydric acid at 110°C. After lyophilization, the hydrolysates were migrated on a TLC plate and autoradiographed. After standardization with amino acids colored by ninhydrin, it appeared that Tax was phosphorylated on serine residues in both insect and mammalian expression systems (Figure 1b).

Tax is phosphorylated on serines 106 and 293

Tryptic peptide maps of ^{32}P-labeled Tax indicated that two major peptides were phosphorylated in Cos and SF9 cells. In spite of the presence of additional peptides in the baculovirus-expressed Tax, this system has the advantage of yielding high levels of protein that then permit direct sequencing. The ^{32}P-labeled peptides were purified by high pressure liquid chromatography (HPLC) and sequenced. Among six peptides, two of

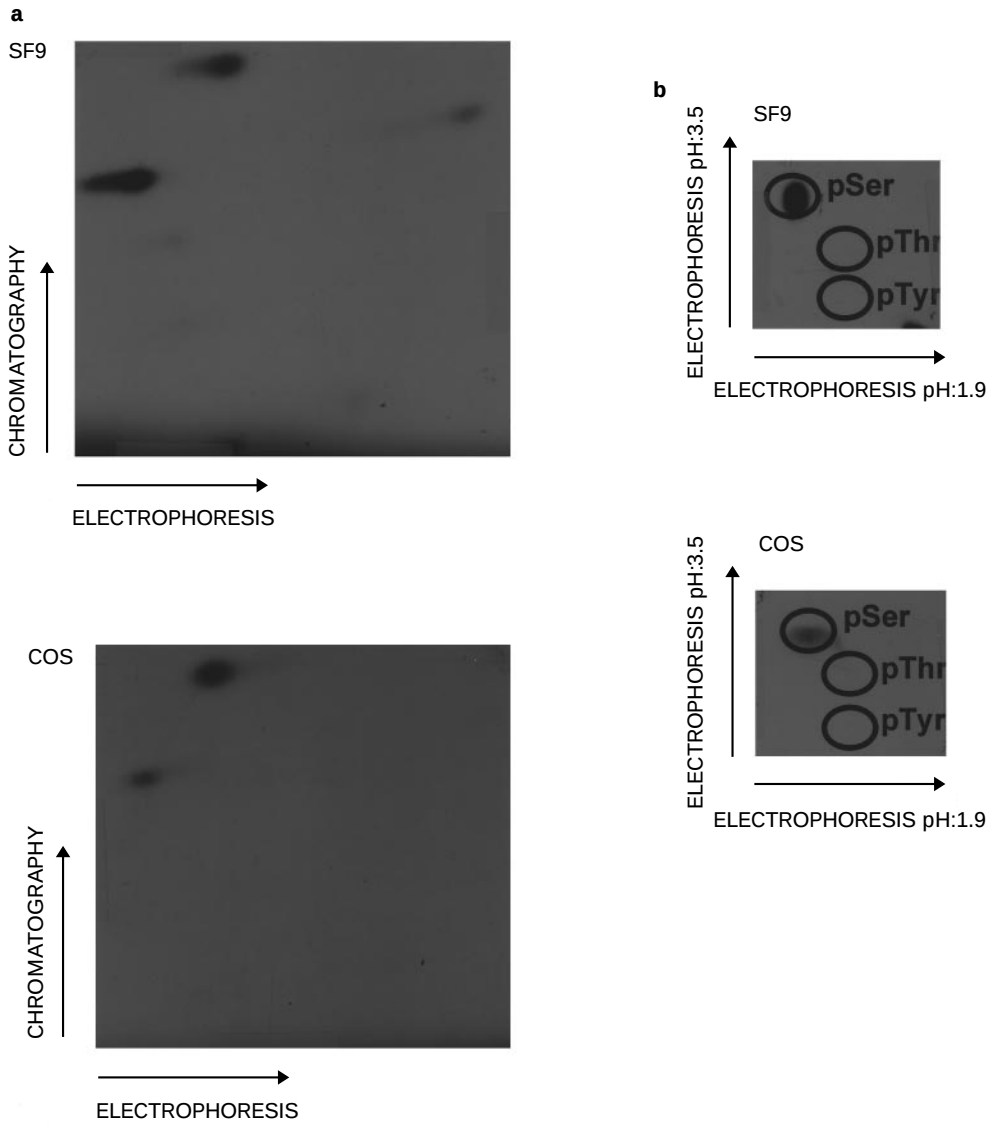

Figure 1 (**a**) Phosphopeptide maps of the Tax protein expressed in SF9 or Cos cells. The Tax protein was expressed in Spodoptera frugiperda (SF9) cells using the vAcTax baculovirus vector, labeled with ^{32}P inorganic orthophosphate and purified by selective precipitation. After tryptic digestion, the peptides were analysed by two-dimensional thin-layer chromatography. To analyse the phosphorylation in Cos, the cells were transfected with the pSGTax effector plasmid and labeled with ^{32}P inorganic orthophosphate. The Tax protein was immunoprecipitated, digested with trypsin and analysed by two-dimensional TLC. (**b**) Two-dimensional TLC of acid-hydrolyzed Tax protein expressed in SF9 or Cos cells. The Tax protein was expressed in SF9 and Cos cells and purified as described in **a**. The Tax protein was next hydrolyzed in chlorhydric acid at 110°C for 1 h. The hydrolysates were spotted on a TLC plate and analysed by two-dimensional electrophoresis. Standard amino acids (pSer, pThr and pTyr) were added to the hydrolysates and colored with ninhydrin

them (Ala98-Arg110 and Ile289-Lys296) yielded the highest ^{32}P counts. To determine whether these peptides were targets for phosphorylation, serines 106 and 293 were substituted with alanines by site-directed mutagenesis of the *tax* gene. Expression vectors encoding wild-type Tax (pSGTax) and each of the two Ser→Ala substitutions were transfected in Cos cells. The proteins were metabolically labeled with ^{32}P, immunopurified and analysed by tryptic peptide mapping. The Ala106 substitution resulted in the disappearance of one phosphopeptide (Figure 2a, pSGSer106) whereas the Ala293 mutation abolished the other one (Figure 2a, pSGSer293). The simultaneous substitution of serines 106 and 293 induced the disappearance of the two phosphopeptides (Figure 2a, pSGSer106+293). The lack of labeled peptide on the autoradiography could be due to the instability of the mutant proteins or to incomplete translation. Therefore, the wild-type and the mutant Tax proteins were metabolically labeled with of a mixture of ^{35}S methionine and cysteine in Cos cells. The Tax proteins were then immunoprecipitated and migrated on a denaturing polyacrylamide gel (Figure 2b). As a control for specificity, the cells were also transfected with an empty pSG5 plasmid. None of these mutations had any effect on the level of Tax expression or on its stability. A similar experiment performed on D17 (canine osteosarcoma) and primary rat embryo fibroblasts (Ref) cells gave similar results (data not shown).

In parallel to these experiments, a series of mutations were introduced in other sites which were putative candidates for phosphorylation (at residues

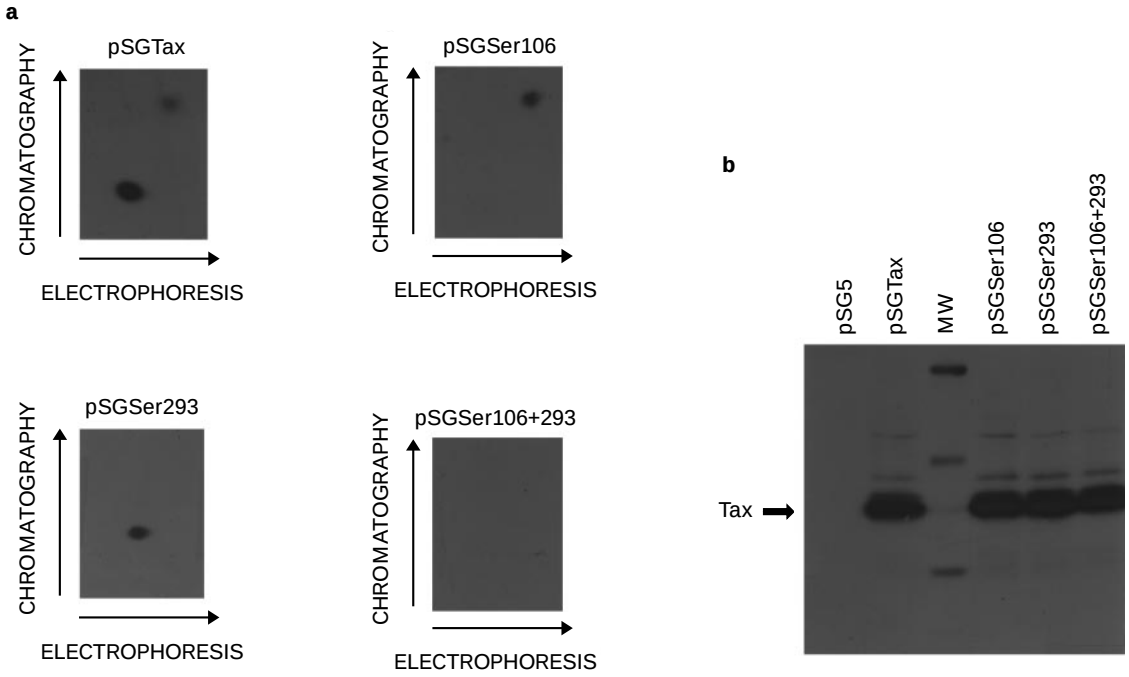

Figure 2 (**a**) Phosphopeptide maps of the mutated and wild-type Tax proteins in Cos cells. The plasmids encoding the wild-type (pSGTax) and mutated (pSGSer106, pSGSer293 and pSGSer106+293) *tax* genes were expressed in Cos cells by transient transfection and labeled with ^{32}P orthophosphate. The tryptic phosphopeptide maps were then performed on the corresponding immunopurified proteins. (**b**) Immunoprecipitation of the wild-type and mutated Tax proteins. Cos cells were transfected with plasmids pSGTax, pSG5, pSGSer106, pSGSer293 and pSGSer106+293 and labeled with ^{35}S methionine and cysteine. The Tax proteins were then immunoprecipitated, migrated on a denaturing polyacrylamide gel and autoradiographied. The molecular weight markers (MW) are 30 kDa (bottom), 44 kDa and 69 kDa (top)

77, 104, 111, 116, 187 and 265). All these constructs yielded phosphopeptide patterns similar to wild-type Tax (data not shown).

We conclude therefore that the substitutions of both serines 106 and 293 prevent the phosphorylation of Tax on two major labeled peptides.

cdc2 and MAP kinases phosphorylate serines 106 and 293 in vitro

It appeared that serines 106 and 293 are the major phosphorylated sites in BLV Tax in insect and mammalian cells. In order to further support the location of these sites, we determined the kinase which could phosphorylate serines 106 and 293. Therefore, the wild-type Tax protein was expressed in bacteria using the pTIT vector under the control of the T7 RNA polymerase promoter (Studier *et al*., 1990). The Tax protein was immunopurified and incubated with different kinases in the presence of inorganic ^{32}P-orthophosphate. The reaction mixtures were then analysed by TLC after tryptic digestion. It appeared that the cdc2 kinase efficiently phosphorylated two peptides containing either serine 106 or serine 293 (Figure 3). These two peptides co-migrated with those obtained from SF9 and Cos cells. In contrast, the $p44^{mpk}$ mitogen-activated protein kinase (MAP) had a marked preference for residue 293 but also phosphorylated serine 106 with a reduced efficiency (Figure 3). Two other kinases, PKC and PKA, were not able to efficiently phosphorylate the serine 293 residue but a weak reaction was observed on site 106 (data not shown). Finally, the casein kinase II was completely inactive on both serine residues.

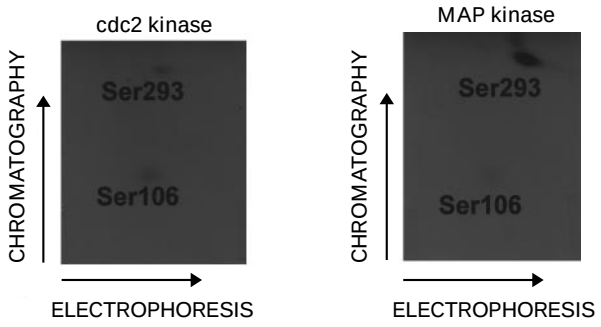

Figure 3 *In vitro* phosphorylation of Tax by cdc2 and MAP kinases ($p44^{mpk}$). The wild-type Tax protein was expressed in bacteria using the pTIT vector under the control of the T7 RNA polymerase promoter. The Tax protein was next immunoprecipitated with a mixture of monoclonal antibodies and incubated with different kinases in the presence of inorganic [^{32}P] orthophosphate. After tryptic digestion, the reaction mixtures were analysed by TLC

In conclusion, the cdc2 and MAP kinases are able to phosphorylate serines 106 and 293 in an *in vitro* reaction. It thus appears that these two serine residues are targets for phosphorylation both *in vitro* and in mammalian cells. These data further underline the importance of these two sites for Tax phosphorylation.

Mutation of serines residues 106 and 293 does not alter transactivation

Phosphorylation has been described to be essential for a series of mechanisms involved in the regulation of

transcription (Hunter and Karin, 1992). To address the effect of the serine substitutions on transactivation by Tax, expression vectors encoding wild-type Tax (pSGTax) and each of the mutants (pSGSer106, pSGSer293 and pSGSer106+293) were co-transfected into Cos cells together with the pLTRCAT reporter. This plasmid contains the BLV promoter cloned upstream of the chloramphenicol acetyltransferase gene (CAT). Forty-eight hours post-transfection, the cells were harvested and the CAT activities were determined from their lysates. The Tax mutants yielded CAT activities similar to those obtained with the wild-type protein (Figure 4). As a control for background levels, transfection of the pSG5 plasmid did not yield significant CAT activities. Different amounts of effector and reporter plasmids used in Cos, D17 or Ref cells gave similar results (data not shown). It appeared from these transient transfection experiments that phosphorylation at serines residues 106 and 293 is not required for transactivation by Tax.

An inhibitor of Tax phosphorylation does not alter its transactivation potential

Phosphorylation of Tax at serines 106 and 293 thus appears dispensable for transcriptional activation. To confirm these data, we developed another experimental protocol based on the use of chemicals able to inhibit phosphorylation of Tax. Therefore, Cos cells were co-transfected with plasmid pSGTax to express the wild-type Tax protein and the pLTRCAT reporter. The transfected cells were then cultivated in the presence of a series of chemicals including W7 (N-(6-aminohexyl)-5-chloro-1-naphtalenesulfonamide), TFP (trifluoperazine) and H7 (1-(5-isoquinolinylsulfonyl)-3-methylpiperazine dihydrochloride). Twenty-four hours post-transfection, they were metabolically labeled overnight with either ^{32}P inorganic phosphate or a mixture of ^{35}S methionine and cysteine. Then, the cells were harvested and the Tax protein was immunoprecipitated from their lysates (Figure 5). It appeared that, under the experimental conditions used, the amounts of ^{35}S-labeled Tax protein were not altered after incubation with the different chemicals (Figure 5a). In contrast, the W7 calmodulin antagonist, but not TFP or H7, strongly decreased the incorporation of ^{32}P into Tax (Figure 5b) indicating that W7 does not alter Tax expression but modulates its phosphorylation. The CAT activities were next determined from cells cultivated in the presence or in the absence of that chemical. It appeared that the incubation with the W7 calmodulin antagonist yielded CAT activities similar to the wild-type levels obtained in the absence of any chemical. Transfection of different amounts of pSGTax effector and pLTRCAT reporter plasmids gave similar results (data not shown). We conclude that it is possible to affect Tax phosphorylation without concomitant effect on its transactivation function. These data further support our transient transfection experiments using Tax mutants substituted on serines 106 and 293.

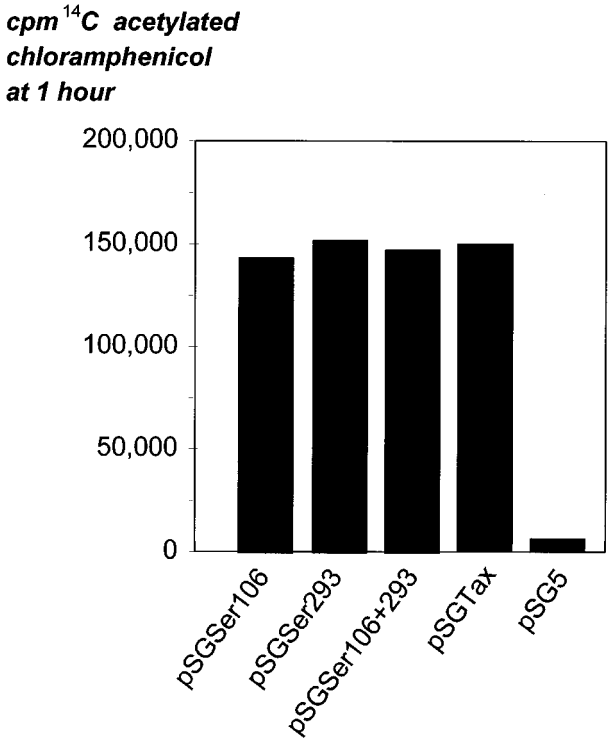

Figure 4 Transactivation by the wild-type and mutated *tax* genes. Five micrograms of the different effector plasmids (pSGTax, pSGSer106, pSGSer293, pSGSer106+293 or the pSG5 control) and 2 μg of pLTRCAT reporter were transfected into D17 cells by the calcium phosphate co-precipitation procedure. CAT activities were determined 48 h post-transfection. The ^{14}C-acetylated chloramphenicol forms were counted at regular intervals to determine the linear range of the reaction. Mean values of three independent measurements performed at 1 h are represented

Phosphorylation at both serines 106 and 293 is required for Tax-induced transformation in vitro

The BLV Tax transactivator protein is also known to be involved in cell transformation (Willems *et al*., 1990). Indeed, Tax cooperates with Ha-ras to transform primary rat embryo fibroblasts (Ref) that are then capable of inducing tumors in thymusless nude mice. To investigate the role of phosphorylation at serines 106 and 293 in the oncogenic potential of Tax, vectors expressing the mutants (pSGSer106 or pSGSer293) were co-transfected with the Ha-*ras* oncogene in Ref cells. Three days post-transfection, the cells were directly injected into nude mice. Mutation of one of the two phosphorylated serines (either Ser106 or Ser293) slightly increased the oncogenic potential of wild-type Tax (Figure 6, compare pSGSer106 and pSGSer293 with pSGTax). The mean volume of the tumors at one month post-injection increased by 50–70% where 100 was wild-type. In contrast, simultaneous mutation of both serines into alanine residues completely destroyed the oncogenic potential of Tax (Figure 6, pSGSer106+293). The mean tumor volume after one month was similar to the background levels obtained with Ref cells cotransfected with the Ha-*ras* oncogene and the control plasmid pSG5. Although it was unable to cooperate with Ha-*ras*, the Ser106+293 mutant was expressed as efficiently as the wild-type Tax (Figure 2b and data not shown). We conclude that single substitution of the phosphorylation sites does not affect primary cell transformation whereas their

concomitant mutation completely destroys Tax oncogenic potential.

To further gain insight into the role of phosphorylation into the oncogenic potential of Tax, the serines 106 and 293 were substituted with aspartic acid. Indeed, it could be that the negative charge rather than the phosphate group itself is required for transformation. Therefore, both serine residues 106 and 293 were mutated in aspartic acid and inserted in the pSG5 vector. The resulting plasmid, called pSGTax2xAsp, was co-transfected into Ref cells together with the pSV_2neoEJ vector. The transfected cells were next analysed for their ability to induce tumors after injection into nude mice. It appeared that the Tax2xAsp mutant very efficiently induced tumors with increased growth potential compared to the wild-type levels (mean tumor volume of 4553 mm^3 at 1 month *versus* 1639 for wild-type). We conclude that the substitution of serines 106 and 293 with aspartic acid maintains Tax oncogenic potential in primary cultures.

Mutation of serines 106 and 293 does not alter viral replication in vivo

To analyse the effect of the serine mutations on viral replication, we used provirus 344 proven to be infectious and pathogenic *in vivo* (Willems *et al*., 1994) to construct three recombinant proviruses (pBLVTax106, pBLVTax293 and pBLVTax106+293) harboring the *tax* mutants. The different proviruses were first analysed in cell culture in transient co-transfection assays together with the pLTRCAT reporter plasmid. It appeared that the substitutions on serines 106 and 293 yielded CAT activities similar to the wild-type levels (Figure 7a). Since the wild-type and recombinant proviruses yielded similar CAT activities, we conclude that phosphorylation at serines 106 and 293 is not required for transactivation by

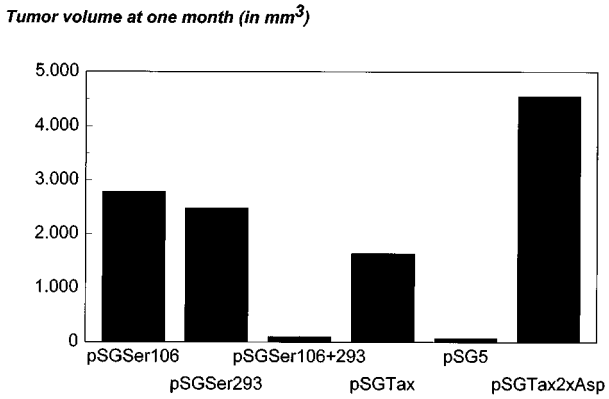

Figure 6 Oncogenic potential of the wild-type and mutated Tax proteins. Primary rat embryo fibroblasts (Ref) were transfected with plasmids containing the wild-type (pSGTax) or the mutated *tax* genes (pSGSer106, pSGSer293, pSGSer106+293, pSGTax2xAsp) and the empty vector (pSG5) were co-transfected together with the pSV_2neoEJ construct that encodes the Ha-*ras* oncogene. Forty-eight hours post-transfection, the cells were collected, washed with PBS and injected subcutaneously into thymusless nude mice. A total of four mice in two independent experiments were injected for each *tax* gene. The tumor volume was calculated by the ellipsoid formula: $4/3\ \Pi\ a\ b^2$ where a and b are respectively the length and the width of the tumor. The mean values among all the tumor volumes are indicated in mm^3

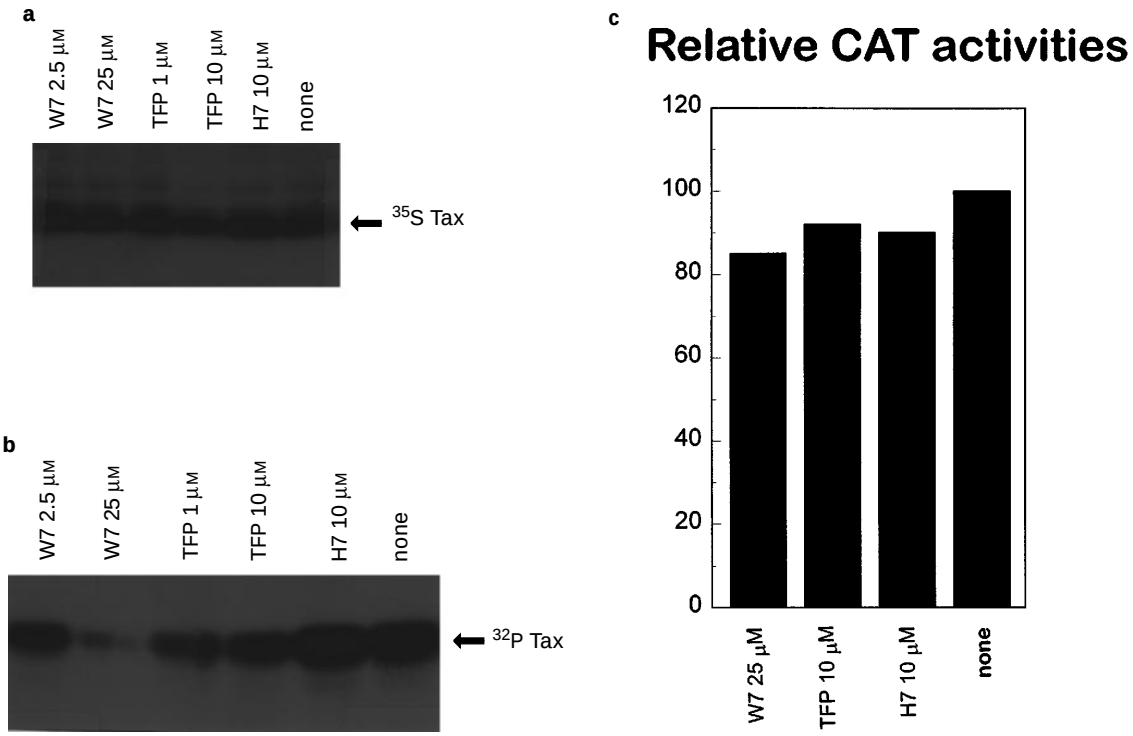

Figure 5 The W7 calmodulin antagonist inhibits phosphorylation of Tax but its transactivation potential. Cos cells were transfected with plasmid pSGTax and the pLTRCAT reporter. The transfected cells were next cultivated in the presence of chemicals: W7, trifluoperazine (TFP) or H7. Twenty-four hours post-transfection, half of the cells were metabolically labeled overnight with either a mixture of ^{35}S methionine and cysteine (**a**) or ^{32}P inorganic phosphate (**b**) and the Tax protein was immunoprecipitated from their lysates. The CAT activities were determined from the other half of the transfected cells cultivated in the presence or in the absence of chemical (**c**). The background levels obtained after transfection of the pSG5 plasmid represented 2% of the CAT activities. The data are the mean values of at least three independent experiments

Tax. These results confirm the CAT activities obtained after transfection of the *tax* genes cloned into the pSG5 expression vector (see Figure 4).

The behavior of the mutated proviruses was next studied *in vivo*. A series of eight sheep were injected intradermally with wild-type (plasmid pBLVIX which contains provirus 344) or recombinant (pBLVTax106, pBLVTax293 and pBLVTax106+293) viruses. All three recombinant proviruses were infectious as demonstrated by serology and PCR (data not shown and Figure 7b). The phosphorylation of serines 106 and 293 is thus not required for *in vivo* viral infectivity. To assess the efficiency of viral replication, the proviral loads were measured by semi-quantitative polymerase chain reaction (PCR) (Figure 7b). Cell lysates were prepared from peripheral blood collected by jugular venipuncture at three months post-infection. The *tax* gene sequences were amplified from these lysates by 22 cycles of PCR. Under these conditions, amplification of viral sequences is semiquantitative in comparison with serial dilutions of the positive control (sheep M11 at 1 year post-infection 1×, 10×, 100×). The amount of viral sequences produced by the recombinant viruses were similar to wild-type virus levels (compare sheep M258, M270, M296, M104 and M480 to M11, M292 and M293). This experiment was repeated at 6 months post-infection and gave similar results (data not shown). As a control, the PCR-amplified *tax* sequences were directly sequenced to verify that the specific mutations were still present in these *in vivo* propagated viruses (data not shown). This demonstrated that the viruses harboring the mutations were not wild-type revertants. Since the proviral loads were similar in all animals, it appears that the substitution of serines 106 and 293 does not impair viral propagation *in vivo*.

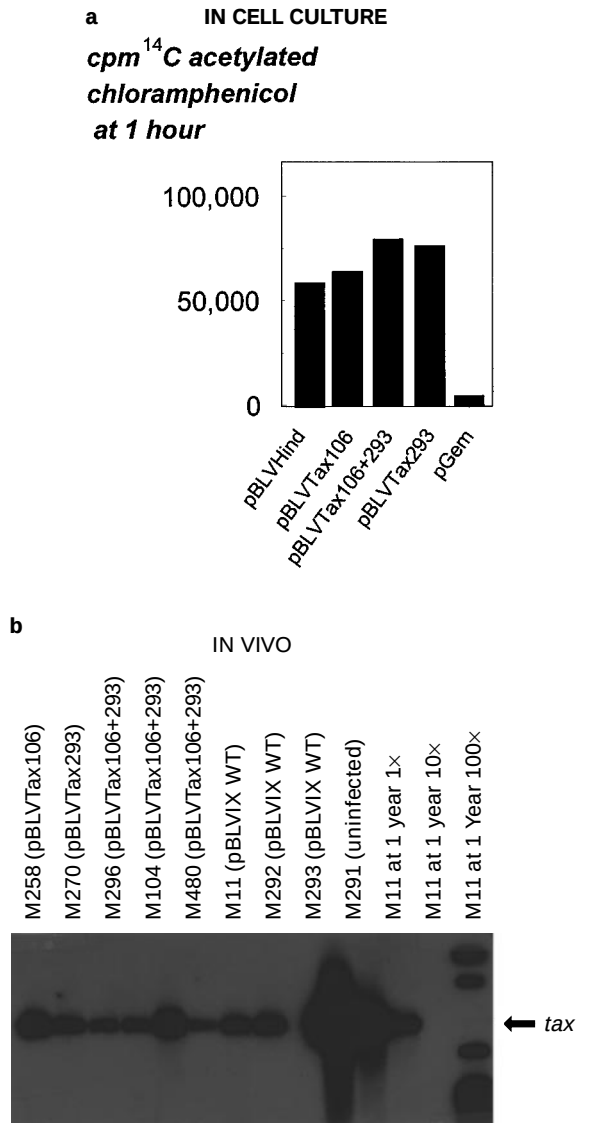

Figure 7 Transactivation potential and replication of wild-type and mutated proviruses. (**a**) Transactivation by the wild-type and mutated *tax* genes. Five micrograms of the different effector plasmids (pBLVHind that harbors the wild type 344 provirus, pBLVTax106, pBLVTax293 and pBLVTax106+293) and 2 μg of pLTRCAT reporter were transfected into D17 cells by the calcium phosphate co-precipitation procedure. As a control, plasmid pGem was analysed in parallel. CAT activities were determined 48 h post-transfection. Mean values of three independent experiments are represented as ^{14}C-acetylated chloramphenicol at 1 h of incubation. (**b**) *In vivo* replication of the proviruses mutated in the *tax* gene. Plasmid DNA containing the different mutant viruses (pBLVSer106, pBLVSer293, pBLVSer106+293) and the corresponding wild-type provirus (pBLVIX) were injected into sheep. At 3 months post-injection, the proviral loads were estimated by semi-quantitative PCR using oligonucleotides flanking the *tax* gene. After PCR, the samples were analysed by Southern blot hybridization using a *tax* probe. As a control for semi–quantitative amplification, serial dilutions of the wild-type provirus were amplified in parallel using a blood lysate from sheep M11 1 year post-infection. MW are the molecular weight standards (1 kb ladder, Promega). This experiment was performed at least in triplicate

Discussion

We have previously shown that it is possible to dissociate the transactivation and immortalization functions of Tax by mutations in the zinc finger structure (Willems *et al*., 1992). These mutants have indeed lost their capacity to activate viral expression directed by the LTR but are capable to immortalize primary cells. In this report, we have characterized mutations that destroy Tax oncogenic potential without concomitant loss of transactivation function. We have indeed demonstrated that two residues of the Tax transactivator protein, serines 106 and 293, are phosphorylated *in vitro*, in insect cells and in mammalian cells. When these serines are mutated into alanines or into aspartic acid residues, the Tax protein still activates LTR-directed gene transcription but does not transform primary cells. There is a bunch of literature concerning the role of phosphorylation in the regulation of transcription. Much less data are available concerning the interplay between phosphorylation, transactivation and oncogenic potential. Inhibition of phosphorylation has been shown to enhance the transformation activity of oncogenic proteins. For example, alteration of a cyclic AMP-dependent protein kinase phosphorylation site in the c-Fos protein augments its transforming potential (Tratner *et al*., 1992). Similarly, the phosphorylation sites in the amino-terminal domain of c-Myc modulate its oncogenic potential (Henricksson *et al*., 1993). Indeed, cells expressing a chicken c-Myc harboring a mutation of Thr61/Ser65 have an increased growth potential in soft agar compared to wild-type transfectants. It thus appears that c-Myc function is negatively

regulated by phosphorylation. In contrast, the oncogenic potential of c-Jun requires phosphorylation on serines 63 and 73 (Smeal *et al*., 1991). In this case, however, the transactivation function of c-Jun in cooperation with Ha-ras is lost. To our knowledge, the sole example where inhibition of phosphorylation impedes transformation without affecting transcriptional activation is provided by the E7 protein of human papillomavirus type 16 (Firzlaff *et al*., 1991). Serine to alanine mutations that changed the casein kinase II phosphorylation site drastically reduced the ability of E7 to co-transform primary cells with *ras* whereas its transactivation capability of the E2 promoter remained unaffected. We provide here an example where the transforming potential of a retroviral transactivator requires phosphorylation of two serine residues independently of its transactivation function. The analogies between the Tax and E7 proteins is even strengthen by the fact that the negative charge provided by an aspartic acid residue restores the oncogenic potential of both transactivators. This observation could indicate that phosphorylation by itself is not required for transformation which would then only require a net negative charge at these sites. Alternatively, the substitution with negatively charged residues would mimic a phosphorylated serine and allow transformation of Ref cells. In this case, the phosphorylation of Tax could be a pathway which could allow the modulation of its oncogenic potential. Therefore, it would be of particular interest to analyse the pathogenicity induced by a BLV provirus harboring aspartic acid residues at positions 106 and 293. Such a mutant could be particularly leukemogenic in the sheep animal model.

It should be recalled here that our transformation assay is based on the cooperation of BLV Tax with the Ha-ras oncogene in rat embryo fibroblasts. This transformation assay, which was extensively used in the past in different systems, has recently been shown to present some limits. Indeed, a HTLV Tax mutant (M703), which was deficient in the CArG transactivation pathway, was unable to transform Ref cells but efficiently immortalized T lymphocytes in culture (Akagi *et al*., 1997; Matsumoto *et al*., 1997). However, the immortalized cultures exhibited an aberrant phenotype of CD8+ instead of CD4+ cells. There is thus no strict correlation between the Ref transformation assay and the immortalization of T lymphocytes in culture. Similarly, it is possible that the defect of the BLV Tax phosphorylation mutant in the Ref transformation assay reflects cell target specificity but not oncogenicity *in vivo*. Of note, in contrast to HTLV Tax, both the wild-type and mutant BLV Tax proteins were unable to activate the CArG pathway.

In insect cells, the BLV and HTLV-I Tax transactivators have been shown to be phosphorylated (Nyunoya *et al*., 1988; Chen *et al*., 1989). This post-translational modification appears to be conserved through the evolution since HTLV-I Tax is also phosphorylated in human lymphocytic cell lines (Fontes *et al*., 1993). We have shown in this report that BLV Tax is also phosphorylated in insect and in mammalian cells, and that serines 106 and 293 appear to be the only residues which are phosphorylated in an *in vitro* kinase reaction. The conservation of this post-translational modification allowed us to map the phosphorylated sites within the Tax protein. The identification of the phosphorylation sites was based on the incorporation of ^{32}P in the tryptic peptides obtained from the Tax protein expressed in SF9 cells. Mutation of these two residues abrogated the incorporation of ^{32}P within the tryptic peptides as shown by thin layer chromatography of Tax expressed in mammalian cells. To further support these data, a series of mutations were introduced in other sites that were target candidates for phosphorylation. We mutated the *tax* gene at serines 77, 111 and 265 because the peptides corresponding to these residues gave minor signals after the HPLC column. A serine to alanine mutation was also introduced at residue 104 because tryptic digestion did not differentiate between serines 104 and 106. In addition, since tyrosine phosphorylation is very inefficient in insect cells, the two tyrosines of Tax (residues 116 and 187) were also substituted with alanine. All these constructs yielded phosphopeptide patterns similar to wild-type (data not shown). It should be mentioned here that the mutation of serine 106 into alanine within Tax also modifies the Rex protein which is translated from an alternative open reading frame on the same messenger RNA. However, this phenylalanine to leucine substitution does not seem to alter Rex function since recombinant viruses harboring the mutation exhibit a wild-type behavior *in vivo*.

Phosphorylation of Tax does not alter its transactivation capacity in cell culture. This is supported not only by transient transfection experiments of mutant *tax* genes but also by the use of a calmodulin antagonist (W7). Indeed, this chemical inhibits Tax phosphorylation but does not alter transactivation of the LTR promoter. In contrast, trifluoperazine, another calmodulin antagonist having another spectrum of inhibition does not alter Tax phosphorylation and its transactivation function in cell culture. Similarly, inhibition of the protein kinase C pathway with H7 does not modulate Tax phosphorylation. We can thus speculate that a calmodulin-dependent pathway is required for efficient phosphorylation of Tax but is dispensable for transactivation. However, we should mention here that W7 also inhibits PKC and that H7 interferes with the cAMP and cGMP pathways (Hidaka *et al*., 1981). Therefore, we should be cautious about the interpretation of data based on the use of chemicals that are not entirely specific. In any case, by using the W7 calmodulin antagonist, it is possible to abrogate Tax phosphorylation without affecting its transactivation function. This observation further supports our data based on the use of Tax mutants substituted on serines 106 and 293.

Phosphorylation has been shown to modulate the nuclear localization of a transcription factor (Hunter and Karin, 1992). Likewise, the substitution of serines 106 and 293 could modify the subcellular localization of Tax. Since the mutants are competent for transcriptional activation, one could assume that the nuclear localization of Tax is not altered by the mutations in the phosphorylation sites. Indirect immunofluorescence staining did indeed not unravel significant differences among the wild-type and mutant Tax proteins (data not shown). To strengthen these observations, the wild-type and mutated Tax proteins were also fused to the green fluorescent protein (GFP)

that allows direct examination under the fluorescence microscope without prior fixation. All the GFP-Tax proteins were present mainly in the nucleus but also to a lesser extent in the cytoplasm of Cos, Ref and Hela cells (data not shown). It thus appears that the subcellular localization of Tax is not modified by the mutations in the phosphorylation sites. However, it is possible that its association with cellular factors is altered leading to a defect in oncogenicity. Further colocalization studies by confocal microscopy are currently under way.

As a model for the human T-cell leukemia virus type I, the infection of sheep by BLV provides a model to analyse viral infectivity, replication and leukemogenesis *in vivo*. We have shown here that proviruses recombinant for the Tax phosphorylation sites exhibit wild-type behavior when injected into sheep. The proviral loads within the infected sheep are indeed similar to the wild-type levels at three and six months post-injection. This observation provides a strong argument for the absence of phosphorylation requirements to ensure transactivation. Indeed, we have previously shown that the infectious potential of BLV proviruses requires a functional Tax protein (Willems *et al*., 1993). In fact, a provirus (clone 395) exhibits a tenfold reduction in transactivation activity due to the presence of two mutations in the *tax* gene (two glutamine codons are substituted with lysine residues). This virus is not infectious *in vivo* when injected into sheep. In contrast, when a functional *tax* gene is provided, the recombinant provirus becomes infectious and replicates at wild-type levels *in vivo* (Willems *et al*., 1993). Since the proviruses recombinant for the Tax phosphorylation sites 106 and 293 propagate at wild-type levels *in vivo*, it appears that essential genes such as Tax and Rex are not affected by these mutations. Altogether, these data demonstrate that LTR-directed transactivation and viral replication *in vivo* do not require the phosphorylation of Tax on serines 106 and 293. In addition, the oncogenic potential of Tax, as measured by a transformation assay of primary Ref cells in cooperation with Ha-ras, is dispensable for infectivity and propagation *in vivo*. It thus appears that the *in vitro* oncogenic potential of Tax is not required for viral persistence in sheep. Whether Tax phosphorylation is required for the induction of leukemia or for cell target specificity is currently unknown. The availability of proviruses harboring a *tax* gene that lacks oncogenic potential as measured by an *in vitro* cell transformation assay provides a very convenient tool to analyse its role in leukemogenesis *in vivo*.

Materials and methods

Phosphopeptide maps of the Tax protein

The Tax protein was expressed in Spodoptera frugiperda (SF9) cells using the vAcTax baculovirus vector as described by Chen *et al*. (1989). SF9 cells were collected 48 h after infection and incubated for 1 h in phosphate-free TC100 medium supplemented with 5% dialyzed foetal calf serum (FCS, Gibco), followed by incubation with 300 μCi/ml of ^{32}P-labeled inorganic orthophosphate (Amersham) for 4 h. The cells were then washed three times in phosphate-buffered saline (PBS) containing 20 μg/ml sodium orthovanadate. After centrifugation, the cells were lysed in a buffer containing 20 mM sodium phosphate pH 7.6, 0.5 M NaCl, 0.5% Tween 20, 20 μg/ml sodium orthovanadate, 1 mM PMSF and 6 M guanidinium chlorhydrate. The concentration in guanidinium chlorhydrate was progressively reduced to 1.5 M allowing the selective precipitation of Tax. The Tax protein was recovered by centrifugation for 1 h at 34 000 *g*, resuspended in 50 mM ammonium bicarbonate and digested with 1 μg/μl trypsin (Promega) for 20 h at 37°C. The reaction was then incubated for 2 h in the presence of additional trypsin (0.2 μg/μl) to ensure complete digestion of Tax. After lyophilization, the protein pellet was resuspended in 20 μl TLC buffer pH 1.9 (formic acid 2%-acetic acid 7.8%). After a brief centrifugation in a microfuge, the supernatant was spotted on a TLC plate (Merck). The sample was then electrophoresed for 40 min at 1.5 kV in TLC buffer pH 1.9 (apparatus type Hunter Thin Layer Electrophoresis HTLE-7000, C.B.S.). After drying, the TLC plate was chromatographied in the second dimension in a buffer containing n-butanol 37.5%/pyridine 25%/acetic acid 7.5% during 15 h and autoradiographied.

To analyse the phosphorylation in mammalian cells, 300 000 Cos cells were transfected with 5 μg of effector plasmids pSGTax (Willems *et al*., 1990), pSGSer106, pSGSer293 and pSGSer106+293 using the calcium phosphate procedure (Promega) and cultivated for 20 h at 37°C in a 5%/95% CO_2/air atmosphere in minimal essential medium (MEM, Gibco) supplemented with 10% FCS, sodium pyruvate 1 mM, L-glutamine 2 mM, penicillin (100 U/ml) and streptomycin (100 μg/ml). The medium was replaced by MEM medium without phosphate supplemented with 5% dialyzed FCS and cultivated for 2 h. After washing, the cells were incubated overnight in the same medium containing 1 mCi/ml ^{32}P-labeled inorganic orthophosphate. After three washes with PBS, the cells were lysed in 1 ml NET buffer (50 mM Tris pH 8.0, 150 mM NaCl, 0.5% Nonidet P-40, EDTA 5 mM, SDS 0.5%) containing 20 μg/ml sodium orthovanadate and 1 mM PMSF (phenylmethylsulfonyl fluoride). The Tax protein was immunoprecipitated with two monoclonal antibodies (5A5 and 6A7), a polyclonal serum directed towards mouse immunoglobulins and protein A CL-4B (Pharmacia). After 20 h incubation at 4°C, the immune complexes were washed three times in RIPA-I buffer (150 mM NaCl, 50 mM Tris pH 7.2, 1% Triton X100, 1% sodium deoxycholate) and two times in RIPA-II (50 mM NaCl, 50 mM Tris pH 7.2). The immunopurified Tax protein was then resuspended in 50 mM ammonium bicarbonate, digested with trypsin and spotted on a TLC plate. The sample was then electrophoresed, chromatographied in two dimensions and autoradiographied.

Two-dimensional thin-layer chromatography of acid hydrolyzed Tax protein

The purified Tax protein was resuspended in 1 ml chlorhydric acid 5.7 M and incubated at 110°C during 1 h. After lyophilization, the hydrolysates were resuspended in 10 μl of TLC buffer pH 1.9 containing standard amino acids and spotted on a TLC plate. The amino acids were electrophoresed at 1.5 kV during 20 min in TLC buffer pH 1.9. After drying, the samples were migrated in the second dimension at 1.3 kV in TLC buffer pH 3.5 (5% acid acetic acid, 0.5% pyridine). Before autoradiography, standard amino acids were visualized with 0.25% ninhydrin (Sigma) in acetone after incubation at 65°C during 15 min.

Identification of the phosphorylation sites

The Tax protein was expressed in SF9 cells, labeled with ^{32}P orthophosphate, purified and digested with trypsin. The peptides were then resuspended in 200 μl trifluoroacetic

acid (TFA) and loaded on an inverse phase HPLC column type 4. The samples were migrated in a gradient formed by two solvents a (0.1% TFA) and b (0.1% TFA in 70% acetonitrile) at a flow rate of 1 ml/min. After the HPLC, the samples were resuspended in TLC buffer pH 1.9 and one tenth of them was migrated on a TLC plate at 1.5 kV during 40 min in the HTLE-7000 apparatus. After autoradiography, the fractions that contained labeled peptides were lyophilized, resuspended in 100% TFA and sequenced by the Edman degradation procedure using the Applied Biosystem 477A and 120A apparatus.

Site-directed mutagenesis of the phosphorylation sites

The serine codons at positions 106 and 293 were mutated by site-directed mutagenesis of the pGemLOR plasmid that contains the *tax* gene cloned into pGem7 (Willems *et al*., 1990). Therefore, primers containing the appropriate mutation were used: Ser106 5′-CCGGTTTCACTTGCCCCCTTC-3′, Ser293 5′-GGACTTGATGCCCCCTTAAAA-3′ and their corresponding complementary oligonucleotides (Ser106c and Ser293c). A first round of PCR allowed the amplification of each half of the *tax* gene using the SP6 and T7 primers located in the pGem7 plasmid. After agarose gel electrophoresis, the DNA fragments were purified using the Sephaglas kit (Pharmacia). A second round of PCR using the SP6 and T7 oligonucleotides allowed to reconstitute the *tax* gene harboring the selected mutation. The mutated genes were introduced in the pSG5 plasmid (at the *Xho*I and *Bam*HI sites) and sequenced to ensure for the presence of the substitution and the lack of Taq DNA polymerase errors. The resulting plasmids (pSGSer106 and pSGSer293) were used to construct the hybrid vector pSGSer106 + 293 using the *Eco*RI site located within the *tax* gene. A similar strategy was used to construct plasmid pSGTax2xAsp in which the serines 106 and 293 were substituted with aspartic acid instead of alanines.

To construct the proviruses mutated in the *tax* gene, the infectious and tumorigenic provirus 344 was used (Willems *et al*., 1994). Therefore, the 3′ proviral end of plasmid pBLVSal (that corresponds to pBLV344 with a *Sal*I-*Sal*I deletion of the 3′ cellular flanking sequences) was site-directed mutagenized by a two step PCR-based strategy. To mutagenize codon 106, the primers used were X31 (5′-TGGAAAGAACTAACGCTG-3′) with Ser106c and Ser106 with Ser293Bc (5′-TTTTAAGGGTGCATCAAGTCT-3′) to amplify the first and the second half of the *tax* gene, respectively. The *tax* gene was reconstituted by a second round of PCR using the X31 and Ser293Bc oligonucleotides. To mutate codon 293, the primers used were Ser106 with Ser293Bc and Ser293B (complementary to Ser293Bc) with SP6 to amplify the first and the second half of the *tax* gene, respectively. The *tax* gene was reconstituted by a second round of PCR using the Ser106 and SP6 oligonucleotides. The mutated proviral sequences were cloned into plasmids pGem7 or pCRII (In Vitrogen) to generate pGemTaxB106 and pCRTax293. The mutated genes were sequenced to ensure for the presence of the substitution and the lack of Taq DNA polymerase errors. The resulting plasmids (pGemTaxB106 and pCRTax293) were used to reconstitute the provirus using the *Xba*I and *Sal*I sites to generate pBLVTax106 and pBLVTax293. The provirus pBLVTax106 + 293 that contains a substitution at the level of both serines 106 and 293 was constructed using the *Eco*RI site located within the *tax* gene.

Immunoprecipitation of the wild-type and mutated Tax proteins

The pSGTax, pSG5, pSGSer106, pSGSer293 and pSGSer106 + 293 plasmids were transfected into Cos cells by the calcium phosphate procedure. Twenty hours post-transfection the cells were cultivated for 2 h in the presence of MEM medium without methionine and cysteine containing 5% dialyzed FCS. The cells were labeled overnight with 100 μCi/ml of a mixture of ^{35}S-methionine and cysteine (Translabel, ICN). The Tax proteins were then immunoprecipitated, migrated on a 12.5% polyacrylamide-SDS (sodium dodecylsulfate) gel and autoradiographied. The molecular weight markers on Figure 2 (MW) are 30 kDa (bottom), 44 kDa and 69 kDa (top) (Amersham).

In vitro *kinase assays*

The Tax protein was expressed in HMS174 bacteria essentially as described by Studier *et al*. (1990). In brief, the pTITTax plasmid (Willems *et al*., 1992) that contains the *tax* gene cloned downstream of the T7 RNA polymerase promoter was introduced into HMS174 bacteria and cultivated in liquid M9 minimal essential medium containing 50 μg/ml ampicillin. Exponentially growing cultures of the transformed bacteria were then infected with bacteriophage CE6 which encodes the T7 RNA polymerase (at a multiplicity of infection of 10). After 2 h, the bacteria were lysed and the Tax protein was immunopurified as described above. After purification, the Tax protein was incubated in the presence of different commercial kinases: cdc2 and MAP kinases (UBI), PKC and CKII (Boehringer) and PKA (Promega). Kinase reactions were performed as described by the manufacturers in the presence of 2 μCi of [^{32}Pγ]ATP and 10 μM cold ATP. The Tax protein was then digested with trypsin and analysed by two-dimensional TLC.

CAT assays

Five micrograms of the different effector plasmids and 2 μg of pLTRCAT reporter were transfected into 300 000 Cos, D17 or Ref cells by the calcium phosphate co-precipitation procedure. The pLTRCAT plasmid contains the BLV LTR cloned upstream of the chloramphenicol acetyltransferase gene. Forty-eight hours post-transfection, CAT activities were determined from the cell lysates. The ^{14}C-acetylated chloramphenicol that migrated into the Econofluor (NEN) phase was counted at regular intervals. At 1 h of incubation, the reaction was still in the linear range. The data represent the mean values of at least three independent experiments (the standard deviations being less than 10%).

To inhibit Tax phosphorylation a series of chemicals from Sigma were added to the culture medium: an inhibitor of protein kinase C, H7 (1-(5-isoquinolinylsulfonyl)-3-methylpiperazine dihydrochloride at a concentration of 10 μM) and two calmodulin antagonists, W7 (N-(6-aminohexyl)-5-chloro-1-naphtalenesulfonamide at concentrations of 2.5 or 25 μM) and TFP (trifluoperazine at concentrations of 1 or 10 μM).

In vivo *replication of the proviruses mutated in the* tax *gene*

One hundred μg of plasmid DNA containing the wild-type provirus (pBLVIX) and the different mutants (pBLVTax106, pBLVTax293, pBLVTax106 + 293) were injected into sheep as previously described (Willems *et al*., 1993). In brief, 100 μg of proviral DNA were mixed with 200 μl DOTAP (N-(1-(2,3-dioleoloxyl) propyl)-N,N,N,-trimethyl- ammoniummethylsulfate, Boehringer) in 1 ml HBS (HEPES 20 mM, NaCl 150 mM, pH 7.4) and injected intradermally in the back of BLV-free sheep. The animals were maintained under controlled conditions at the National Institute for Veterinary Research (Uccle, Belgium). Their serum was collected at regular intervals and analysed for the presence of BLVgp51 specific antibodies by the ELISA procedure (Portetelle *et al*., 1989). At 3 months post-injection, the proviral loads were estimated as previously described (Willems *et al*., 1994). In brief, blood

samples (500 μl aliquots) were mixed with an equal volume of lysis buffer (0.32 M sucrose, 10 mM Tris-HCl pH 7.5, 5 mM $MgCl_2$, 1% Triton X-100). After 20 s centrifugation, the pellets were resuspended in 1 ml lysis buffer. This step was repeated twice. The samples were then resuspended in 200 μl PCR buffer (50 mM KCl, 2.5 mM $MgCl_2$, 10 mM Tris-HCl pH 9, 0.1% Triton X-100) and incubated with 1 μl proteinase K (5 mg/ml) for 1 h at 50°C. The digestions were stopped by boiling the sample for 5 min. Ten μl aliquots were amplified by PCR in the presence of 0.2 mM each dNTPs, 200 ng primers and 1 U of Taq DNA polymerase (Boehringer). Two oligonucleotides were used: PCRTD (5′-AACGACAAAATTATTTCTTGTC-3′) and PCRTC (5′-CCTGCATGATCTTTCATACAAAT-3′). The reaction mixtures were overlaid with two drops of mineral oil, denatured for 5 min. at 94°C and amplified by 22 cycles (30 s at 94°C, 30 s at 57°C and 1 min at 74°C). After PCR, the samples were analysed by Southern blot hybridization using a *tax* probe. As a control for semi-quantitative amplification, serial dilutions of the wild-type provirus were amplified in parallel using a blood lysate from sheep M11 1 year post-infection. MW are the molecular weight standards (1 kb ladder, Promega). These experiments were performed in triplicate at 3 and 6 months after seroconversion.

Transformation of primary rat embryo fibroblasts

Primary rat embryo fibroblasts (Ref) were prepared from F344 rats at day 14 of pregnancy (IFFA CREDO) and cultivated for 2 days in MEM medium supplemented with 10% FCS, sodium pyruvate 1 mM, L-glutamine 2 mM, penicillin (100 U/ml) and streptomycin (100 μg/ml). The plasmids (5 μg) containing the wild-type (pSGTax) or the mutated *tax* genes (pSGSer106, pSGSer293, pSGSer106 + 293 and pSGTax2xAsp) and the empty vector (pSG5) were co-transfected together with the pSV_2neoEJ construct that encodes the Ha-ras oncogene into 2×10^6 Ref cells. As a control for equal expression of the Tax protein, an immunoprecipitation and a CAT assay was performed in parallel (data not shown). Forty-eight hours post-transfection, the cells were collected, washed with PBS and injected subcutaneously into thymusless nude mice. A total of four mice in two independent experiments were injected for each *tax* gene construct. The tumor volume was calculated by the ellipsoid formula: 4/3 Π a b^2 where a and b are respectively the length and the width of the tumor. The mean values between all the tumor volumes are indicated in mm^3.

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