Evidence that protein kinase A activity is required for the basal and *tax*-stimulated transcriptional activity of human T-cell leukemia virus type-I long terminal repeat

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Abstract The present study was undertaken to investigate the role of protein kinase A (PKA) in the control of human T-cell leukemia virus type-I (HTLV-I) long terminal repeat (LTR) expression, since this issue is still controversial. For this purpose we employed two human T-cell lines; the Jurkat cells in which long exposure to diBu-cAMP severely down-regulated the catalytic subunit of PKA (PKA-C), and H-9 cells in which such exposure markedly increased PKA-C level. Transient transfection assays revealed that addition of diBu-cAMP 1 h before or after transfection profoundly increased HTLV-I LTR directed CAT expression and synergistically enhanced its stimulation by the viral transactivator *tax* gene product in both cell lines. However longer exposure to diBu-cAMP before transfection reduced LTR-CAT expression to below its basal level and completely abolished its stimulation by *tax* in Jurkat cells, and this diBu-cAMP inhibitory effect could be abrogated by co-transfection of a PKA-C expressing vector. By contrast, in H-9 cells, this long exposure to diBu-cAMP continued enhancing LTR-CAT expression and its *tax*-mediated transactivation, and this stimulatory effect of diBu-cAMP could be diminished by the PKA-specific inhibitor *N*-[2-(*p*-bromocinnamylamine)ethyl]-5-isoquinolinsulfonamide (H-89). Notably, in the absence of diBu-cAMP treatment H-89 reduced LTR-CAT expression to below its basal level and prevented its stimulation by *tax* in both cell lines. Together these findings indicate not only that cAMP-activated PKA stimulates HTLV-I LTR expression and its transactivation by *tax*, but even in the absence of PKA activating signals the basal HTLV-I LTR expression as well as its stimulation by *tax* are both dependent on a basal PKA activity.

Key words: HTLV-I; *tax*; LTR-CAT; Protein kinase A (PKA); Dibutyryl-cAMP; H-89

1. Introduction

Human T-cell leukemia virus type-I (HTLV-I) is the etiologic agent of adult T-cell leukemia (ATL) and tropical spastic paraparesis/HTLV-I associated myelopathy (TSP/HAM) and apparently of certain other clinical disorders [1]. However, after infection the virus enters into a latent state, rendering the infected individuals asymptomatic seropositive carriers, of whom only a small proportion eventually develop an HTLV-I related disorder 20–40 years later [1]. During this latency a very low viral gene expression can be detected in the infected T-cells of such carriers [2]. In contrast, TSP/HAM is characterized by a high virus expression which seems to account for most of the pathoimmunological manifestations of this syndrome [2]. Furthermore, the HTLV-I transactivator *tax* gene product is widely considered to account for the viral leukemogenicity due to its capacity of activating a wide variety of cellular genes and acting as an oncogene in cell culture and transgenic mice [1,3,4]. Therefore, generating an HTLV-I related disorder plausibly depends on viral gene activation in the latently infected carriers. Understanding the control system of HTLV-I expression, which involves complex interactions between viral and cellular regulatory elements is essential for having an insight into the mechanisms underlying the latency and reactivation of this virus.

Tax transactivates the viral promoter, located in the 5′-long terminal repeat (LTR), via three repeats of incomplete but highly homologous 21 bp enhancer sequences. Each of these repeats consists of three functionally distinct domains designated A, B and C. Domain B, which is crucial for this transactivation, contains a TGACGT motif that is highly homologous to the cAMP responsive element (CRE), found in a variety of genes that are regulated by the cAMP-activated protein kinase A (PKA) through members of the CREB/CREM/ATF transcription factor family [5]. This domain binds, indeed, members of this family [5–10], which mediate the effect of *tax* on HTLV-I gene expression [3,11–13]. Domains A and C are G-C rich and seem to control the relative level of this *tax* effect [8,14–16]. Although it is generally accepted that *tax* exerts this effect through interaction with members of the CREB/CREM/ATF family, there are indications that certain other cellular proteins may also mediate *tax* interaction with the 21 bp repeats [15,17–19].

However, despite intensive research the mechanism of *tax* effect on the viral LTR is unclear yet, mainly because of the numerous contradictory data related to *tax* interaction with these transcription factors. For instance, certain studies demonstrate that tax can interact only with CREB [16,20,21], whereas others prove that *tax* can interact with every member of the CREB/CREM/ATF family [7,9,22–24]. Certain investigators claim that *tax* can exert a stimulatory effect through these proteins only when CRE is in the context of the viral 21 bp repeat [8,11,12,16,20], whereas others demonstrate that *tax* can activate also other CRE containing genes [3,23–25]. Certain reports show that *tax* enhances the binding of these transcription factors to the HTLV-I CRE merely by facilitating their dimerization [21–24], while others provide evidence that *tax* binds to the DNA together with the dimerized transcription factors and stabilizes the DNA-protein complex through interaction with the A and C domains of the 21 bp repeats [14,16]. While certain data indicate that after binding to the DNA/protein complex *tax* employs its own transcrip-

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tion activating domain [3,11,26,27], others demonstrate that it requires the CREB binding protein (CBP) for exerting its transactivatory effect [23,28]. Particularly intriguing is the conflict over the very fundamental question of whether or not PKA activity is required for these interactions of *tax*. While certain data show that *tax* can activate both HTLV-I LTR and other CRE containing promoters independently of PKA activity [3], others prove that *tax* can use unphosphorylated CREB/CREM/ATF factors only for transactivation of the HTLV-I LTR but needs their phosphorylation by PKA for transactivation of other CRE containing genes [23,28]. Furthermore, there are studies showing that *tax* depends on PKA activity even for activating the viral LTR [12,13].

cAMP activates PKA by binding to its two regulatory subunits (PKA-R) and dissociating, thereby, the two catalytic subunits (PKA-C), which then migrate to the nucleus, where they phosphorylate the target transcription factors [29,30]. Extended exposure to high intracellular cAMP level results in either up- [31,32] or down-regulation [33–36] of PKA-C, depending on the cell type. In order to shed more light on the role of PKA in controlling HTLV-I expression we decided to investigate the effect of this PKA-C modulation on virus expression. For this purpose we used two different human T-cell lines; the Jurkat cells in which long exposure to diBu-cAMP severely down-regulated PKA-C and H-9 cells in which such exposure markedly increased PKA-C level. Our results indicate that activated PKA enhances the basal expression of HTLV-I LTR as well as its transactivation by *tax*. Furthermore, we provide here evidence that even in the absence of PKA activating signals the basal HTLV-I LTR expression as well as its stimulation by *tax* are both dependent on a basal PKA activity.

2. Materials and methods

2.1. Cells

Two human T-cell lines, Jurkat and H-9, were employed in this study. The cells were grown in RPMI-1640 medium supplemented with antibiotics and 10% fetal calf serum. All ingredients were purchased from Kibbutz Beith Haemek, Israel.

2.2. Plasmids, DNA electroporation and CAT assay

Plasmid containing the complete HTLV-I LTR linked to the bacterial CAT reporter gene was a gift of Dr. K. Khazaie from the German Cancer Center, Heidelberg, Germany. Plasmid expressing the HTLV-I *tax* gene under CMV promoter was provided by Dr. I.S.Y. Chen from UCLA, Los Angeles, USA and plasmid expressing PKA-C was provided by Prof. Y. Shaul from the Weizman Institute, Rehovot, Israel. Plasmids were introduced into the cells by electroporation and CAT activity was assayed as previously described [37]. The amount of the various transfected plasmids in the indicated combinations were: LTR-CAT 20 μg, the tax expressing vector 10 μg and the PKA-C expressing vector 10 μg per transfection.

It was important to estimate the effect of pre-treatments with the PKA activator diBu-cAMP, or with the PKA-specific inhibitor *N*-[2-(*p*-bromocinnamylamine)ethyl]-5-isoquinolinsulfonamide (H-89) [38], on the plasmid uptake by the transfected cells. For this purpose we prepared an ^{3}H-labeled LTR-CAT plasmid by growing the host bacterial cells in the presence of 0.8 μCi/ml ^{3}H-thymidine (60 mCi/mmol, DuPont New England Nuclear Product) for overnight before isolating the plasmid (51 000 cpm/μg DNA). DNA uptake by the pre-treated cells was estimated by including 1 μg labeled plasmid in parallel identical transfection mixtures and 48 h after transfection the cells were lysed and the DNA was pelleted from each lysate and assayed for its radioactivity. The supernatant of each lysate was assayed for its protein content by Bio-Rad Protein Assay Reagent and the plasmid uptake was normalized to the protein concentration of the respective extracts.

2.3. PKA activity assay

PKA activity was assayed in the cell extracts by the Pierce Colorimetric PKA Assay Kit (SpinZime Format, Rockford, IL, USA) with cAMP present in the reaction mixture, according to the manufacturer's instructions. This activity was normalized to the protein concentration of each extract.

3. Results

3.1. Regulation of PKA-C by diBu-cAMP in Jurkat and H-9 cells

In the present study we were interested to estimate the effect of PKA-C up- and down-regulation on the control of HTLV-I expression. Therefore, we characterized the pattern of cAMP-mediated regulation of PKA-C in various T-cell lines. This was done by assaying PKA activity in cell extracts after various times of exposure to 1 mM diBu-cAMP. The enzymatic activity was measured in a reaction mixture containing cAMP and, therefore, it represented the total level of the PKA-C in the extract (i.e. the PKA-C that was still bound to PKA-R and the dissociated PKA-C). The results shown in Fig. 1 demonstrate a clear difference in the pattern of PKA-C regulation between Jurkat and H-9 cells. While extended exposure to diBu-cAMP results in a time-dependent decline of the total PKA-C level in Jurkat cells, it induces a continuous increase of this level in H-9 cells. Therefore, we choose this pair of cell lines for our subsequent experiments.

3.2. Effect of diBu-cAMP and H-89, added after DNA transfection, on LTR-CAT expression and on its transactivation by tax

In initial experiments HTLV-I LTR-CAT was transfected either alone (Fig. 2A and C) or together with the *tax* expressing vector (Fig. 2B and D) into Jurkat (Fig. 2A and B) or H-9 (Fig. 2C and D) cells. Where indicated, diBu-cAMP was added 2 h after transfection, whereas the PKA-specific inhibitor H-89 was added 1 h earlier to ensure its uptake before diBu-cAMP. These experiments demonstrated that diBu-

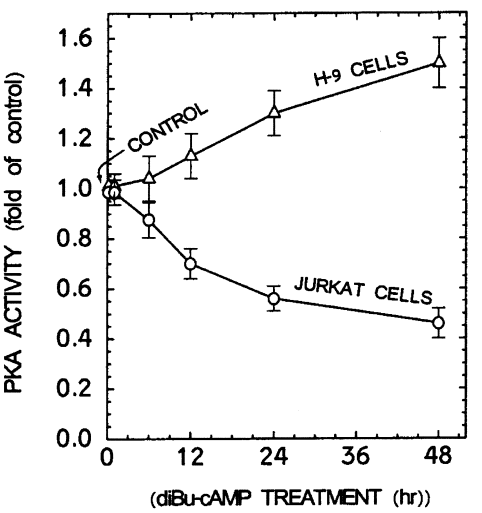

Fig. 1. Up- and down-regulation of PKA-C in H-9 and Jurkat cells. H-9 (△) and Jurkat (○) cells were incubated with 1.0 mM diBu-cAMP for the indicated times and their extracts were analyzed for PKA activity as described in Section 2. The enzymatic activity is presented as fold of the activity measured in the respective diBu-cAMP untreated control cells. The data represent the average of four replicates ± S.D.

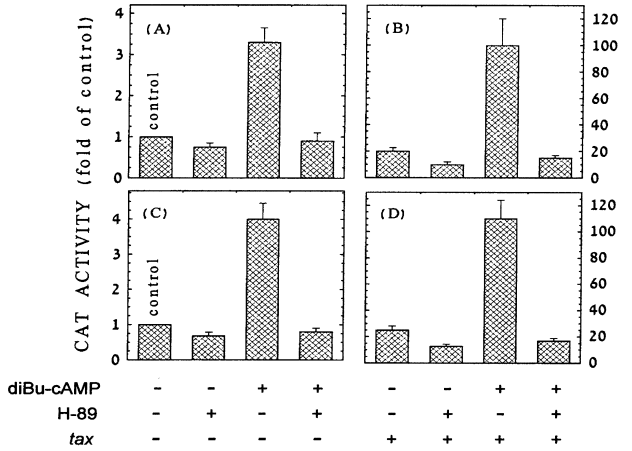

Fig. 2. Effect of diBu-cAMP and H-89 added after transfection on LTR expression and its transactivation by *tax*. Jurkat (A and B) and H-9 (C and D) cells were transfected with LTR-CAT alone (A and C) or together with *tax* expressing vector (B and D) and then treated with diBu-cAMP and/or with H-89 as described in the text. CAT activity was normalized to protein concentration of the respective extract and expressed as fold of the respective control cells. The data represent the average ± S.D. of four repeated experiments with Jurkat cells and five repeated experiments with H-9 cells.

cAMP stimulated LTR-CAT expression in both cell lines and that this stimulation was abolished by H-89. The inhibitor alone moderately reduced the LTR basal expression. They also showed that diBu-cAMP synergistically enhanced the transactivatory effect of *tax*. Interestingly, the level of LTR-CAT expression in the presence of the combination of *tax* and diBu-cAMP was reduced by H-89 to markedly below the level observed with *tax* alone. Moreover, even in the absence of diBu-cAMP this inhibitor markedly reduced both the basal LTR-CAT expression as well as its stimulation by *tax*. However, of particular note is that despite the different diBu-cAMP-mediated regulation of PKA-C elicited by these two cell lines, both gave practically the same results under all these experimental conditions. This indicates that addition of diBu-cAMP after transfection is inadequate to demonstrate the effect of the PKA-C up- or down-regulation.

3.3. *Effect of PKA up- and down-regulation before DNA transfection on LTR-CAT expression and on its transactivation by tax*

In the next experiments Jurkat and H-9 cells were exposed to diBu-cAMP for increasing time and then were transfected with LTR-CAT alone or LTR-CAT+*tax*. DiBu-cAMP remained in the medium after transfection to avoid restoration of PKA-C to its initial level. These experiments revealed that diBu-cAMP pre-treatment of Jurkat cells for 1 h before transfection still stimulated the expression of LTR-CAT (Fig. 3A). Moreover, this short pre-treatment synergistically enhanced the activation of this construct by *tax* (Fig. 3B). However, longer diBu-cAMP pre-treatment of these cells gradually reduced LTR-CAT expression to below its basal level (Fig. 3A), and almost completely abolished the stimulatory effect of *tax* (Fig. 3B). By contrast, in H-9 cells, this long pre-treatment stimulated LTR-CAT expression (Fig. 3A) and synergistically enhanced its transactivation by *tax* (Fig. 3B) throughout the entire time range of these experiments.

To check the possibility that these results merely reflect an effect of these pre-treatments on the DNA uptake by the cells we added 1 μg of ^{3}H-labeled LTR-CAT containing plasmid to transfection mixtures similar to those of the experiments shown in Fig. 3 and measured the DNA uptake as described in Section 2. The different samples varied by up to ± 12% from the average uptake determined for each cell line (32 200 cpm/mg extract protein for Jurkat cells and 27 400 cpm/mg extract protein for H-9 cells; data not shown), thus excluding such an artifact.

3.4. *Effect of PKA-C expressing vector on LTR activation by tax in diBu-cAMP pre-treated Jurkat cells*

To determine whether the inhibitory effects of the extended diBu-cAMP pre-treatment observed in Jurkat cells resulted from the PKA-C down-regulation, these cells were pre-treated with diBu-cAMP for 24 h and then transfected with LTR-CAT alone, LTR-CAT+PKA-C, LTR-CAT+*tax* and LTR-CAT+*tax*+PKA-C. DiBu-cAMP remained in the culture medium after transfection to avoid restoration of the intrinsic PKA-C level and to prevent the association of the free cellular PKA-R molecules with the PKA-C molecules expressed by the transfected vector. These experiments demonstrated that restoring PKA activity by the PKA-C expressing vector restored the stimulation of LTR-CAT expression in the diBu-cAMP treated Jurkat cells (Fig. 4A). Moreover, this PKA-C restoration also synergistically enhanced the stimulatory effect of *tax* in these cells (Fig. 4B). Introducing the PKA-C expressing vector into diBu-cAMP untreated cells resulted in lower stimulation of LTR-CAT expression (Fig. 4A) and only moderately enhanced its transactivation by *tax* (Fig. 4B). These lower effects can be explained by assuming that in the absence of diBu-cAMP part of the PKA-C molecules encoded by the exogenous vector are masked by cellular PKA-R molecules.

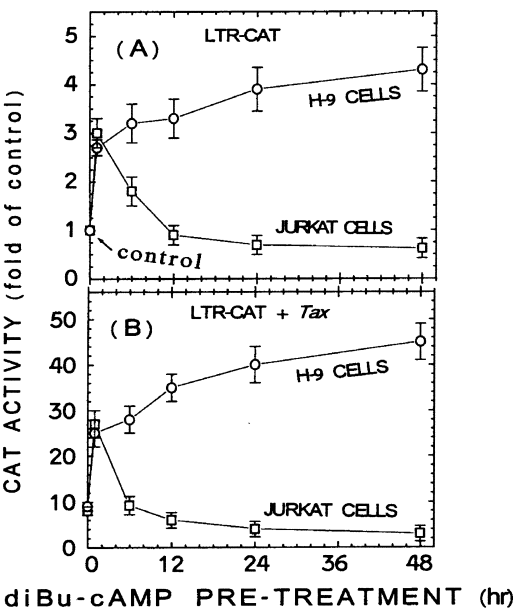

Fig. 3. Effect of extended diBu-cAMP pre-treatment on LTR expression and on its transactivation by *tax*. Jurkat (□) and H-9 (○) cells were pre-treated with diBu-cAMP for the indicated time and then transfected with LTR-CAT alone (A) or together with *tax* expressing vector (B). CAT activity is expressed as fold of the specific activity measured in diBu-cAMP untreated respective control cells transfected with LTR-CAT alone. Each point in the graphs represents the average respective results ± S.D. of five repeated experiments.

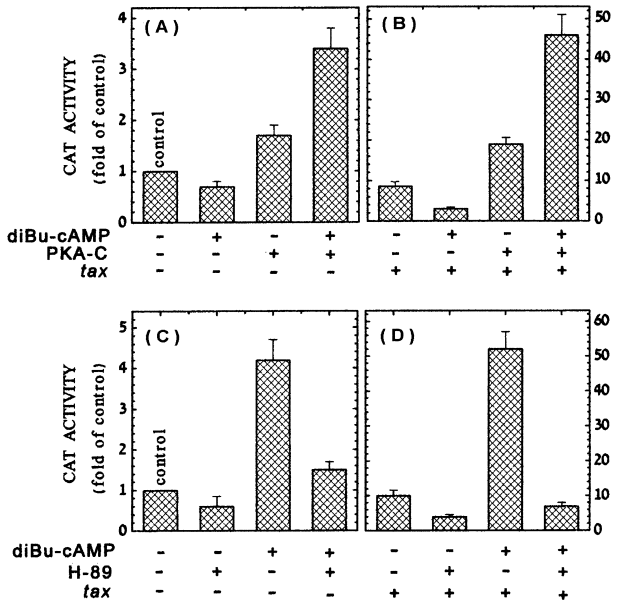

Fig. 4. Effect of diBu-cAMP pre-treatment, PKA-C expressing vector and H-89 on LTR expression and its transactivation by *tax* in Jurkat and H-9 cells. Jurkat (A and B) and H-9 (C and D) cells were pre-treated with diBu-cAMP for 24 h and then transfected with LTR-CAT alone (A and C) or together with *tax* expressing vector (B and D). Where indicated Jurkat cells were co-transfected also with PKA-C expressing vector whereas H-9 cells received the PKA inhibitor H-89 1 h before diBu-cAMP. CAT activity is presented as fold of the control activity measured in diBu-cAMP untreated cells transfected with LTR-CAT alone. The data represent the averages ± S.D. of four repeated experiments.

3.5. *Effect of H-89 on LTR activation by tax in the diBu-cAMP pre-treated H-9 cells*

To examine the postulation that the stimulatory effects of diBu-cAMP pre-treatment observed in H-9 cells resulted from the increasing PKA-C level, these cells were pre-treated with diBu-cAMP in the absence or presence of the PKA inhibitor, H-89 (45 μM), for 24 h before transfection. (H-89 was added 1 h earlier to ensure its penetration into the cells before diBu-cAMP.) DiBu-cAMP and H-89 remained in the culture medium after transfection to avoid subsequent restoration of PKA to its initial status. These experiments demonstrated that H-89 abolished the stimulatory effect of diBu-cAMP pre-treatment on LTR-CAT expression and reduced the expression of this construct in diBu-cAMP untreated cells to below its basal level (Fig. 4C). Moreover this inhibitor strongly lowered the stimulatory effect of *tax* in diBu-cAMP untreated cells and reduced LTR-CAT expression in cells exposed to the combination of diBu-cAMP pre-treatment and *tax* to below the level scored with *tax* alone (Fig. 4D).

4. Discussion

Long exposure to PKA activating signals leads to either down- or up-regulation of the catalytic subunit of PKA, depending on the cell type [31–36]. In attempt to shed more light on the role of PKA in controlling HTLV-I gene expression we used the Jurkat cell line, in which long exposure to diBu-cAMP severely down-regulated PKA-C, and H-9 cell line, in which such exposure profoundly up-regulated this catalytic subunit. In all previous investigations of the effect of cAMP on HTLV-I LTR expression the adenylate cyclase activators were added after DNA transfection [3,5,11–13].

Our finding that both Jurkat and H-9 cells elicited the same response of LTR-CAT expression and its *tax*-mediated transactivation to diBu-cAMP added after transfection proves that such experimental conditions are inadequate for demonstrating the effect of the up- or down-regulation of PKA-C.

Since under such conditions PKA was activated and stimulated the relevant cellular transcription factors in the presence of the reporter LTR construct, the expression of this construct and its transactivation by *tax* were, as expected, equally enhanced in both cell lines. Thus the subsequent up- or down-regulation of PKA-C could have, in these conditions, only a marginal influence on the ultimate level of the reporter enzyme activity, since these PKA-C modulations plausibly became effective only shortly before assaying this activity. This interpretation was further substantiated by our subsequent experiments which readily illustrated the effect of these PKA-C modulations with cells in which the level of catalytic subunit was already up- or down-regulated before transfection.

Pre-treatment of Jurkat cells with diBu-cAMP for 1 h before transfection, still profoundly stimulated LTR expression and synergistically enhanced its transactivation by *tax*, most likely because at this stage the level of active PKA-C was still sufficiently high. However, longer diBu-cAMP pre-treatment before transfection progressively reduced LTR expression and its stimulation by *tax* in a time-dependent kinetics that correlated with that of the PKA-C down-regulation. On the other hand, similar exposure of H-9 cells to diBu-cAMP resulted in a continuously increasing stimulation of LTR expression and synergistic enhancement of its transactivation by *tax*. These data point to a strict dependency of LTR-CAT expression as well as of the *tax* effect on PKA activity. This conclusion is further supported by our observation that the inhibitory effect of the extended diBu-cAMP pre-treatment in Jurkat cells could be reverted by co-transfection of a PKA-C overexpressing vector, whereas the stimulatory effect of this prolonged diBu-cAMP noted in H-9 cells could be blocked by the PKA-specific inhibitor H-89. Moreover, our finding that LTR expression as well as its transactivation by *tax* are markedly reduced by H-89 even in diBu-cAMP untreated cells strongly suggests that both depend on a basal PKA activity existing in the absence of PKA activating signals. This conclusion is consistent with other reports showing that HTLV-I LTR activation by *tax* is much lower in PKA-deficient mutant cells than in their wild-type counterparts [12,38]. Hwang et al. [39] have reached to a similar conclusion for genes involved in catecholamine synthesis. These authors have shown that the expression of these genes is stimulated by PKA activating signals and that in the absence of such signals their basal expression is profoundly reduced by H-89.

It is of interest to discuss the possible roles of CREB and CREM proteins in the outcomes of our present experiments. All CREB isoforms act as activators of the CRE containing genes. Their activity is enhanced by PKA-mediated phosphorylation of their kinase induced domain (KID) and negatively regulated by specific protein phosphatases [30]. CREM isoforms are highly homologous to CREB, especially in their DNA binding and KID domains. However, with the exception of CREM-τ, all other CREM isoforms lack a transcription activating domain and are, therefore, considered to func-

tion as repressors of cAMP regulated genes, either by competing with CREB proteins on binding to CRE or by heterodimerizing with CREB proteins and abolishing, thereby, their transcription activation potential. The ultimate effect of cAMP on various CRE containing genes is thought to depend on the relative affinity of each CRE to each of these isoforms, and on the quantitative ratio between the various CREB and CREM isoforms, which varies in different tissues and cell types [30]. Interestingly, Laurence et al. [23] have shown that binding of certain CREM isoforms (CREM-α and CREM-δ) to the HTLV-I CRE allows *tax* to stimulate the viral LTR expression, although they inhibit the effect of *tax* on other CRE containing genes.

Another important factor involved in cAMP related signalling system is the so-called 'inducible cAMP early repressor' (ICER). This factor is encoded by an mRNA initiated from an internal promoter within the CREM gene. This promoter contains CRE and therefore can potentially be activated by cAMP, although this activation seems to be tissue- and cell type-specific. ICER itself also binds to CRE, but acts to repress a variety of cAMP regulated genes, including its own, after their initial activation by cAMP [30]. Bodor et al. [40] have shown that transfection of ICER overexpressing vector blocks *tax*-mediated transactivation of HTLV-I LTR.

By assuming that the affinity of ICER to CRE is higher than that of the unphosphorylated CREB or CREM proteins but lower than that of their phosphorylated forms, it can be postulated that the extent of LTR expression and of its transactivation by *tax* is modulated by the quantitative ratio between ICER level and the phosphorylated form of CREB/CREM proteins. Bodor et al. [39] have shown that elevated intracellular cAMP level induces ICER synthesis in mature human T-cells. However, although PKA activation by diBu-cAMP might induce ICER formation in both Jurkat and H-9 cells, as long as PKA activity is sufficiently high the phosphorylated CREB/CREM proteins are probably dominant and stimulate LTR expression and its *tax*-mediated transactivation. While this situation remains persistent in the diBu-cAMP treated H-9 cells due to their PKA-C up-regulation, the down-regulation of this subunit in Jurkat cells permits dephosphorylation of CREB/CREM proteins and consequently the inhibitory effect of ICER on the viral LTR may turn to be dominant. Furthermore, as noted before, without PKA activating signal both LTR expression and the effect of *tax* are driven by the basal PKA activity of the cells. Under such conditions there is no ICER in the cells and therefore even the low basal level of CREB/CREM phosphorylation is presumably sufficient to support the LTR activation by *tax*. These hypotheses still require experimental evidence.

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