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Transcription factor AP-4 participates in activation of bovine leukemia virus long terminal repeat by p34 Tax

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ABSTRACT

Three 21bp repeats can be found in the bovine leukemia virus long terminal repeat, which are crucial for the LTR directed gene expression by the *trans* activator protein Tax. Previous studies demonstrated that the major target of the Tax directed activation are the CRE-like elements in the center of these repeats. In this work we report that another motif of the 21bp repeats is also required for the Tax activation. Gel retardation — with the wild type or mutant 21bp repeats — revealed that cellular factors from HeLa cells were specifically bound to the center (CRE-like element) and the 3′ region of the repeats, which contains a CAGCTG consensus AP-4 binding site. *In vivo* analysis using the synthetic 21bp repeats indicated that beyond the consensus CRE-like motif, the AP-4 site is also essential for Tax activation. To determine the role of AP-4 in BLV Tax *trans* activation, we used the AP-4 cDNA in antisense transient assays. In the *in vivo* experiments the antisense AP-4 RNA resulted in strongly decreased Tax activation. On the basis of these results we conclude that AP-4 is a good candidate of cellular factors involved in BLV Tax *trans* activation.

INTRODUCTION

Bovine leukemia virus (BLV) is a B-lymphocytotropic retrovirus of cattle and the etiologic agent of enzootic bovine leukemia (3). BLV is structurally and biologically closely related to human T-cell leukemia virus types I and II (HTLV-I, HTLV-II) (18). Transcription of these viruses is controlled by a *trans* activator protein Tax, encoded in the virus genome (6,20,21). The long terminal repeats (LTRs) contain enhancer elements mediating Tax activation (6,2). The most important of these activators are the 21bp repeats found in three tandem copies in the LTRs. Since Tax does not bind directly to these elements, its activation effect is thought to be realized via interactions with cellular factors bound to the 21bp repeats (14,1). We and others have previously shown that in BLV one of these cellular factors is a member of the CREB/ATF protein family, which can bind to the CRE core sequence in the center of the repeat elements *in vitro* and can activate the transcription *in vivo* (22,11). The other components of the activation complex have yet to be identified.

In this study we attempted to localize sequence elements in the BLV 21bp repeats, which are also essential for Tax activation, and to find the proteins, which interact with these *cis* acting regulatory regions. We demonstrate that a CAGCTG consensus AP-4 binding site — found in the 3′ flanking region of two of the 21bp repeats — is necessary for Tax *trans* activation. In gel mobility shift assays using synthetic 21bp repeats, we could detect specific protein binding to the intact, but not to the mutated AP-4 site. In agreement with the *in vitro* results, destroying the AP-4 site abolished the responsiveness of the BLV promoter to Tax *in vivo*. In cotransfection experiments the antisense AP-4 RNA decreased the level of Tax activation dramatically. These results strongly suggest that beyond the CREB protein, AP-4 may be another transcription factor involved in the regulation of the BLV LTR directed gene expression.

MATERIALS AND METHODS

Plasmid constructs

Plasmids pBLH2CAT, pXB-RS and pBΔ16 have been previously described (5,19,6). For the expression of AP-4 we changed the *Hin*dIII–*Afl*II fragment of pRSVCREB (11) into an *Hin*dIII–*Bam*HI fragment, which included the AP-4 cDNA. The resulting plasmid was called pRSVAP4. The orientation of the *Hin*dIII–*Stu*I subfragment of the AP-4 cDNA in pRSVAP4 was changed, resulting in pRSVAS. pRSVAS was used for the antisense transient assays.

Gel mobility shift assay and preparation of HeLa nuclear extract

HeLa cell nuclear extract was prepared as described by Dignam *et al.* (7). For detection of specific DNA–protein complexes by gel mobility shift assay, the double-stranded oligonucleotides were labeled by [α-^{32}P]dCTP with the Klenow enzyme. For each sample 1–2μg of nuclear extract, 1μg of poly[d(I-C)] and 10^5 cpm probe DNA were mixed in binding buffer (10mM HEPES pH 7.9, 50mM KCl, 1mM EDTA, 1mM DTT, 1% glycerol) in a 10 μl reaction mixture. Samples were incubated for 20 min at room temperature, and loaded on native polyacrylamide gel.

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The electrophoresis was carried out at 150 V for 2.5 hr at room temperature.

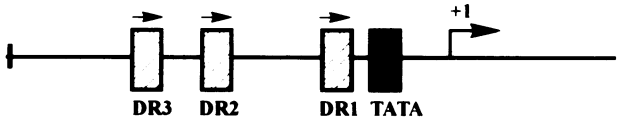
Cell culture, transfection and CAT assay

HeLa cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% foetal calf serum. Transfections were performed by the calcium phosphate procedure. Cells were harvested 48 hours after transfection and one-fifth of the extract was used for CAT assays. CAT activity was determined as have been described previously (9). CAT activity was quantitated by cutting the appropriate spots and determining their radioactivity by LSC.

RESULTS

In vitro protein binding to the AP-4 site in BLV 21bp repeats

The BLV 21bp repeats located in the U3 region of the LTR, show high homology to each other (Fig. 1.). To examine which sequence elements of the repeats can play a role in Tax activation, we synthetised oligonucleotides bearing mutations in different parts of the second repeat element (Fig. 2.). As it has already been shown that the CRE element located in the center of the repeat, is involved in Tax *trans* activation, we focused our investigations only on the nucleotides surrounding the CRE-motif. Oligonucleotide B20 contained the wild type sequence of the repeat. In B22 and B23 we created mutations in the 5′ and in the 3′ regions of the repeat element, respectively. In B24 we mutated the sequences on both sides of the central CRE-motif.



DR1: GAGCTGCTGACCTCACCTGCT

DR2: AAGCTGGTGACGGCAGCTGGT

DR3: CAGACAGAGACGTCAGCTGCC

Figure 1. Schematic structure of the BLV LTR U3 region with respect to the positions of the three 21bp repeats (DR1−3). The individual sequences of the repeats are also shown.

B20 TCGAAAAGCTGGTGACGGCAGCTGGTGGCTAG

B22 TCGAAAAG**GCT**GTGACGGCAGCTGGTGGCTAG

B23 TCGAAAAGCTGGTGACG**GCTCCCCG**TGGCTAG

B24 TCGAAAAG**GCT**GTGACG**GCTCCCCG**TGGCTAG

Figure 2. Nucleotide sequences of the synthetic 21 bp repeats. The nucleotides in boldface correspond to mutations. The core sequence of the central CRE-motif is underlined. The arrows indicate the boundaries of the 21 bp element.

In gel mobility shift assays using the wild type or the mutant oligonucleotides and HeLa cell nuclear extract, we could detect two specific bands with varying intensity (Fig. 3.). We have already previously shown that the lower mobility band corresponded to the CREB protein (11). In oligonucleotide B22, mutations 5′ to the central CRE-motif abolished the binding of the CREB protein but had no effect on the formation of the higher mobility band. The intensity of the higher mobility band correlated well with the mutations in the 3′ flanking region, where we could identify a CAGCTG consensus AP-4 binding site. With oligonucleotides B23 and B24 bearing mutations in the AP-4 site, this higher mobility specific band disappeared.

The AP-4 site is necessary for BLV Tax *trans* activation

To decide whether the detected protein binding to the AP-4 site corresponded to *in vivo* activity, and to elucidate further the role of the AP-4 site in BLV Tax activation, we constructed synthetic LTRs with the above oligonucleotides in pBΔ16. pBΔ16 contained the BLV minimal promoter, the TATA box and the first 21 bp repeat linked to the chloramphenicol−acetyltransferase (CAT) reporter gene. This minimal promoter could not be *trans* activated by Tax. We cloned the synthetic oligonucleotides in two copies upstream of the minimal promoter. The resulting constructs with structure similar to the wild type LTR, were named according to the synthetic oligonucleotides they contained. These clones were cotransfected with a Tax expression plasmid into HeLa cells and their CAT activities were determined. Transfections with each of the plasmids were repeated three times with the same results. As shown in Figure 4, pB20 containing the wild type repeats and pB22 bearing mutations outside of the CRE-motif and the AP-4 site, were capable of responding to Tax.

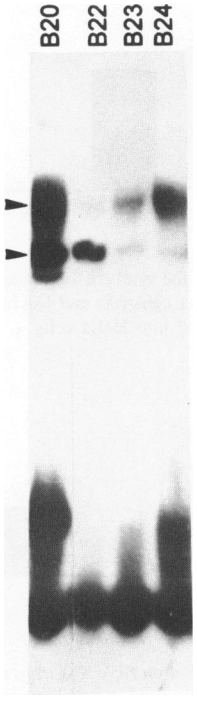


Figure 3. Protein binding to the synthetic 21bp repeats. Gel shift assay was carried out with the ^{32}P-labeled synthetic oligonucleotides (B20, 22, 23, 24) and HeLa cell nuclear extract. The resulting two specific bands are indicated by arrows.

However, in pB23 and pB24 mutations in the AP-4 site eliminated Tax activation. These *in vivo* data, in accordance with the *in vitro* results, made it probable that the protein binding to the AP-4 site, was required for BLV Tax activation.

The *in vivo* involvement of AP-4 in BLV Tax *trans* activation

To obtain more direct evidence about the proposed role of AP-4 protein in Tax activation, we inhibited the production of endogeneous AP-4 in HeLa cells with the pRSVAS plasmid. This plasmid directed the expression of the AP-4 cDNA in antisense orientation under the control of the strong Rous sarcoma virus (RSV) promoter. The transcribed antisense RNA could inhibit the production of the cellular AP-4 protein. In this way Tax activation could be analysed in an *in vivo* system, which had only one component missing. The effect of the antisense AP-4 RNA on Tax activation was examined in cotransfection experiments (Fig. 5, lanes 2–3). In these experiments using increasing amount of pRSVAS, we could detect proportionally stronger decrease in the level of Tax activation. Cotransfection only the RSV promoter instead of the antisense construct under same conditions had no effect on the *trans* activation (data not shown). These results clearly showed that in the lack of the AP-4 protein BLV Tax could not efficiently *trans* activate the BLV promoter.

Next we wished to investigate the effect of the overexpressed AP-4 protein on Tax activation. For this purpose, we cloned the longest known AP-4 cDNA in sense orientation in pRSVAP4. Because this partial AP-4 cDNA did not contain the entire coding sequence of the AP-4 protein, pRSVAP4 directed the production of a protein, which lacked some ten N-terminal amino acids of the whole AP-4 product. Contrary to our expectations, the overexpressed N-terminal deletion AP-4 protein diminished Tax activation in cotransfection experiments (Fig. 5, lanes 4–5).

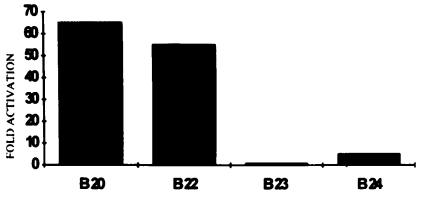


Figure 4. *In vivo* activity of the synthetic oligonucleotides cloned in the plasmid pBΔ16. 3μg of each promoter construct and 1μg of pXBRS coding for the entire BLV Tax were cotransfected into HeLa cells.

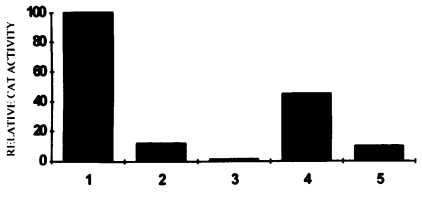


Figure 5. *In vivo* effect of AP-4 on BLV Tax *trans* activation. 3μg of pBLH2CAT containing the BLV LTR were used in each transfection. In Lane 1 pBLH2CAT was activated with 1μg of pXBRS. The activity of this construct was used as a reference and its activity was taken to be 100%. In lanes 2 and 3 pBLH2CAT was cotransfected with 1μg of pXBRS and 1 (lane 2) or 2μg (lane 3) of pRSVAS. Similarly, in lanes 4 and 5 3μg of pBLH2CAT was *trans* activated with 1μg of pXBRS and 1 (lane 4) or 2μg (lane 5) of pRSVAP4.

Taking the above results into consideration we speculate that without the unknown N-terminal domain, AP-4 can not mediate Tax *trans* activation.

DISCUSSION

The Tax responsive elements in BLV and HTLV-I share remarkable homology. Both of them contain 21bp repeats in three tandem copies in the LTRs. Each of the 21bp repeats has a CRE-motif in the middle, serving as binding sites for the CREB/ATF protein family. Several studies demonstrated that members of this protein family that bind to the center of the 21bp repeats, are involved in BLV and HTLV-I Tax activation. Despite the similarity in the enhancer elements, BLV and HTLV-I Tax can not crossactivate each other's promoter. One reason of this may be the differences in the sequences surrounding the CRE-motif in the 21bp repeats.

In this work we investigated the role of the nucleotides adjacent to the central CRE-motif in the BLV repeats. Using synthtetic mutant or wild type 21bp repeats in gel mobility shift assays, we could show evidence that a cellular factor from HeLa cells was specifically bound to the 3′ region of the repeat. In this part we identified a CAGCTG sequence element, which was identical to the consensus binding site of the AP-4 transcription factor. Mutations affecting the CAGCTG motif excluded protein binding. Data obtained from the *in vivo* analyses of the oligonucleotides in HeLa cells, well correlated with the *in vitro* results: the synthetic oligonucleotides, which contained mutations in the putative AP-4 site in the 3′ region, abolished Tax responsiveness of the BLV LTR, while mutation in the 5′ region did not alter Tax activity. Our results suggested that the AP-4 protein played a role in Tax *trans* activation.

AP-4 was first identified as a cellular factor that binds to the SV40 enhancer and activates the viral late gene transcription (15). It has also been shown to activate the hMTIIA gene transcription and participate in the regulation of human proenkephalin expression (4,15). AP-4 belongs to the rapidly growing group of HLH proteins (10). Members of this protein family include enhancer binding proteins and transcription factors involved in differentation and cellular proliferation (8,12,13,16,17). These proteins share a conserved structural motif, referred to as helix–loop–helix (HLH) that mediates DNA binding and dimerization. A striking feature of AP-4 is that beyond the HLH motif it contains two distinct leucine repeat elements, which also direct dimerization and allow the selective complex formation of the ubiquitous AP-4 protein (10).

To verify the involvement of AP-4 in BLV Tax activation, we performed transient assays using the AP-4 cDNA in antisense orientation. In the antisense experiment, we could detect strongly reduced Tax activity. As sequence analysis could not show any significant homology between the nucleotide sequence of AP-4 and other proteins, the effect of the antisense experiment could only result from the decreased level of the cellular AP-4 mRNA. It strongly suggests that in the absence of the AP-4 protein the functional Tax activation complex can not be formed.

Finally, we examined the effect of the overexpressed AP-4 in our transient expression system. Surprisingly, the overproduced AP-4 diminished Tax activation. The explanation for this result, most probably, is in the structure of the AP-4 cDNA. The longest AP-4 cDNA isolated so far and used in our experiments, lacks some regions coding for the N-terminal part of the AP-4 protein

(10). For this reason, the protein overproduced in the transient expression system in HeLa cells, is an N-terminal deletion product. Our data obtained from the above experiments, strongly suggest that the cellular AP-4 forms a multiprotein complex with other cellular factors to mediate Tax activation through the 21 bp repeats. However, when the N-terminal deletion product is expressed in huge amounts compared to the cellular counterpart, it might be assembled in the complex. Due to the lack of the N-terminal domain, which seems to be important for the complex formation and may code for a protein interface, the deletion product disrupts the higher order structure of the complex and makes it unable to respond to Tax activation.

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