Identification and functional characterization of BTas transactivator as a DNA-binding protein

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The genome of bovine foamy virus (BFV) encodes a transcriptional transactivator, namely BTas, that remarkably enhances gene expression by binding to the viral long-terminal repeat promoter (LTR) and internal promoter (IP). In this report, we characterized the functional domains of BFV BTas. BTas contains two major functional domains: the N-terminal DNA-binding domain (residues 1–133) and the C-terminal activation domain (residues 198–249). The complete BTas responsive regions were mapped to the positions −380/−140 of LTR and 9205/9276 of IP. Four BTas responsive elements were identified at the positions −368/−346, −327/−307, −306/−285 and −186/−165 of the BFV LTR, and one element was identified at the position 9243/9264 of the BFV IP. Unlike other foamy viruses, the five BTas responsive elements in BFV shared obvious sequence homology. These data suggest that among the complex retroviruses, BFV appears to have a unique transactivation mechanism.

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Introduction

Foamy viruses (FVs), also known as spumaretroviruses, are members of a subfamily of Retroviridae. FVs represent a distinct family of complex retroviruses and the former have been isolated from several mammalian species, including humans (Achong et al., 1971), nonhuman primates (Bieniasz et al., 1995; Broussard et al., 1997; Herchenroder et al., 1994), and other species (Johnson et al., 1983; Mochizuki et al., 1990; Tobal-Tapiero et al., 2000). Although FVs establish life-long persistent infection in the host, the pathogenic potential of FVs has not been clearly established (Linial, 1999). Unlike other retroviruses, FVs uniquely harbor an internal promoter (IP) in addition to the conventional long-terminal repeat (LTR) (Linial, 1999). The FV genome encodes three structure genes, namely gag, pol, and env, as well as the three regulatory genes, namely tas, bet, and bel-2, that are located between the env gene and the 3′ LTR.

The prototype foamy virus (PFV) Bel1/Tas transactivator acts as a key regulator of gene expression and directly binds to the Bel1 responsive element (BRE) in both the LTR and IP promoters, producing a robust transcriptional transactivation (He et al., 1996). Bel1 consists of at least two functional domains, an N-terminal/central DNA-binding domain (DNA-BD) (residues 88–200) and a C-terminal activation domain (residues 272–300) (He et al., 1993a). In simian foamy virus (SFV), two potent activation domains (residues 1–48 and 272–300) are identified in the Tas protein (Mergia et al., 1993). The Tas responsive elements (TREs) of SFV, SFV-1, SFV-3, and feline foamy virus (FFV) have been identified and are located in the U3 region of viral LTR. A recent study showed that the PFV LTR contains three BREs located at the positions −360/−342, −327/−284, and −116/−89 (Erlwein and Rethwilm, 1993), and the IP contains a minimal Bel1-specific DNA target site located at −166/−140 within the IP.BRE, upstream of the second cap site (Kang et al., 1998). A more precise analysis of the Tas responsive region in SFV-1 and SFV-3 revealed that the 8249/8370 segment of SFV-1 IP and two segments (−1196/−880 and −403/−125) in SFV-1 LTR respond to SFV-1 Tas (Mergia et al., 1992; Zou and Luciw, 1996). Further, two segments (−637/−496 and −496/−180) in SFV-3 LTR that confer Tas responsiveness to a heterologous promoter have been mapped (Renne et al., 1993). In the FFV LTR, two regions with the same responsiveness to a heterologous promoter have been mapped (Renne et al., 2004).

Bovine foamy virus (BFV) is the first nonprimate FV that was isolated in 1983 (Johnson et al., 1983). The genomic organization of
BFV is similar to that of the PFV. The BFV genome contains three open reading frames (ORFs) encoding the gag, pol, and env structural genes and the betas and bet regulatory genes. The betas gene encodes a 249-amino acid (aa) regulatory protein termed BTas (Wang et al., 2009). BTas is a DNA-binding protein that can transactivate both the LTR and IP promoters similarly to PFV Bel1 (Tan et al., 2008; Wang et al., 2009). However, the molecular basis of BTas-mediated transactivation of its own LTR and IP, such as the binding and activation domains of BTas and the precise localization of TRE in the LTR and IP, is still unknown. In this study, we identified a DNA-BD at the N-terminus and a potent transcriptional activation domain at the C-terminus of BTas using deletion mutation analysis. Further, we characterized the binding sequences of BTas on LTR and IP. Several conserved bases in these sequences were found to be crucial for the binding of BTas to its target sites.

**Results**

**Identification of the BTas binding regions on LTR and IP**

Previous studies using deletion mutants and transient expression suggested that LTR.TRE (−380/−140) was important for the transactivation of BTas (Liu et al., 2000). Whether BTas can directly bind to the LTR.TRE (−380/−140) and the precise sequence requirement remains undefined. To identify whether BTas can bind to LTR.TRE (−380/−140) specifically in vitro, the electrophoretic mobility shift assay (EMSA) was carried out. As shown in Fig. 1A, purified BTas can bind to LTR.TRE (−380/−140) DNA probes, and the complexes did not form when unlabeled LTR.TRE was added as competitors. As a negative control, purified GST protein could not bind to the LTR.TRE (−380/−140).

To determine the precise location of BTas the LTR.TRE, a set of LTR segments were synthesized and labeled (Fig. 1B). As shown in Fig. 1A, BTas could bind to two regions (−380/−277 and −310/−140) of LTR.TRE, and the complexes were competed by unlabeled probes.

![Fig. 1. Identify the BTas responsive regions on LTR. (A) EMSA was performed with purified GST or BTas. Digitonin-labeled LTR.TRE (−380/−140, −380/−277, −310/−140, and −239/−170) probes were used. (B) Schematic representation of a series of probes on LTR.TRE. All these probes were labeled by digitonin and then EMSA was performed; the results of EMSA were presented with +/−.](image)

![Fig. 2. Identify the BTas responsive elements on LTR. (A) Sequence alignment the four BTas responsive elements (−368/−346, −327/−307, −306/−285, and −186/−165) on LTR.TRE. (B) EMSA was performed with purified GST or BTas. Digitonin-labeled four BTas binding regions and LTR (−161/−140) (a negative control) were used as the probes.](image)

whereas BTas could not bind to the LTR.TRE (−239/−170) probe. In addition, probes containing regions −310/−277, −310/−257, −239/−140, −200/−140, −161/−140, and −186/−165 were also tested, and the EMSA results were summarized in Fig. 1B. From the above results, a 22-bp BTas responsive element was located in −186/−165. We then performed sequence alignment to determine whether other similar sequences are present in the LTR.TRE (−186/−165). Indeed, conserved bases were found in three other elements (−368/−346, −327/−307, −306/−285) on LTR.TRE (Fig. 2A). To test whether BTas can bind to these four elements separately, we performed EMSA, and the results showed that BTas could specifically bind to all four elements in vitro. As negative controls, purified GST protein could not bind to these elements, and BTas could not bind to the LTR.TRE (−161/−140) element (Fig. 2B).

The IP.TRE has been mapped to the position 9205/9275 (Zhang et al., 2000). To determine the BTas responsive element on IP.TRE, we performed sequence alignment of the IP.TRE and the four BTas responsive elements on the LTR.TRE. A 22-bp element (9243/9264) on IP.TRE exhibited a high homology (82%) to LTR.TRE (−306/−285) (Fig. 3A). The specific binding of BTas to this 22-bp element was subsequently confirmed by EMSA (Fig. 3B).

These results suggested the presence of a conserved “TTA” triplet in the C-terminal of the five BTas responsive elements. To further investigate if this “TTA” triplet is required for BTas binding, we mutated the “TTA” of LTR.TRE (−186/−165) to “CCC” (Fig. 4A), EMSA indicated that BTas did not bind to the mutated −186/−165 (m) probe but only bound to the wide type probe, which was used as a positive control (Fig. 4B). These results suggested that the highly conserved C-terminal “TTA” triplet is required for the binding of BTas to the five BTas response elements.

**Identification of the DNA-binding sequence of BTas protein**

EMSA revealed that BTas could directly bind to the LTR.TRE. To further identify the DNA-binding domain of BTas involved in transactivation, we constructed a series of glutathione S-transferase (GST)-fused terminal deletion mutants of BTas and performed EMSA using purified mutant proteins (Fig. 5A). The results suggested that the BTas DNA-binding domain is located in the N terminal 1−133 aa, as purified BTas mutant containing 27−249 aa could not bind to the LTR.TRE, whereas the BTas mutants containing 1−133 aa and 1−217 aa retained the ability to bind to LTR.TRE, as that observed with the wild-type BTas protein (Fig. 5B). These results indicated that the N-
terminal of BTas is indispensable whereas the 116 amino acids at the C-terminal of BTas are not involved in the binding of BTas to the LTR.

In addition, we used a mammalian expression system to confirm the identified BTas DNA-binding domain in mammalian cells, using BFV LTR-Luc reporter gene. The expression of the reporter gene can be used to assess the DNA-binding capacity of a BTas domain fused to a GAL4 activation domain (AD). A series of pCMV-AD-BTas deletion mutants were constructed and cotransfected with BFV pLTR-luc and pCMV-β-gal into 293T cells. Only marginal luciferase activity was detected in cells cotransfected with GAL4-AD-BTas (27–249 aa) and BFV LTR-luc. In contrast, robust luciferase activity was detected when 293T cells were cotransfected with GAL4-AD-BTas (1–133 aa) and BFV LTR-luc. Further, the induced luciferase activity significantly increased in a dose-dependent manner, whereas GAL4-AD did not show any effect on the Gal4-Luc reporter gene (Fig. 5C). These results mapped the DNA-binding domain of BTas to the N terminal 1–133 aa.

Identification of the minimal activation domain of BTas

To identify the activation domain of BTas, we constructed a series of pCMV-BD-BTas deletion mutants (Fig. 6A) and cotransfected them with pFR-luc (GAL4-luc) and pCMV-β-gal. As shown in Fig. 6B, all N-terminal truncated mutants, including BTas (90–249 aa), BTas (126–249 aa), BTas (167–249 aa), and BTas (198–249 aa), retained full activation ability. The minimal activation domain was mapped to the 198–249 aa of BTas, as C-terminal deletions including BTas (167–234 aa), BTas (198–249 aa), and BTas (167–217 aa) all led to attenuation or loss of the activation ability of BTas. These results suggested that the 198 amino acids at the N-terminal of BTas were dispensable, while the 52 amino acids at the C-terminal were essential for the full activity of BTas. In addition, BTas contains an inhibitory domain at residues 90–127, as the activation ability of BTas (90–249 aa) is similar to that of the wild-type BTas, whereas the activity of BTas (126–249 aa), BTas (167–249 aa), and BTas (198–249 aa) is higher than that of wild-type BTas.

In higher eukaryotes, there are three different sequence motifs in the transcription activation domains: the acidic motif, glutamine-rich motif, and proline-rich motif (Mitchell and Tjian, 1989). Among these, only the acidic activation motif appears to function effectively in the Saccharomyces cerevisiae. To examine whether the activation domain of BTas also functions in yeast cells, we constructed a set of yeast expression plasmids that expressed fusion constructs of GAL4 DNA-BD and BTas mutants. We found that compared to yeast cells transformed with a negative control GAL4-BD, those transformed with GAL4-BD-BTas and GAL4-BD-BTas (198–249 aa) exhibited robust β-galactosidase activity, whereas those transformed with GAL4-BD-BTas (1–133 aa) only exhibited marginal β-galactosidase activity (Fig. 6C). We therefore concluded that BTas contains a
transcriptional activation domain, which is also active in yeast cells, and that this sequence contains an acidic motif.

Discussion

Although the importance of BTas in the transactivation of BFV LTR and IP and the replication of BFV is well established, the mechanisms of BTas function and regulation remain unknown. Eukaryotic transcription factors can be divided into discrete protein domains that either direct the factor to the appropriate DNA target sequence or permit the activation of transcription after binding has occurred. Functional analyses of a series of BTas deletions revealed the presence of at least two key functional domains that include an N-terminal binding domain (1–133 aa) and a C-terminal activation domain (198–249 aa).

The specific DNA-binding activities of several mammalian factors have been localized to relatively small subregions consisting of 60–100 aa. These studies have revealed that a DNA-BD is necessary but not sufficient for transcriptional activation. Three different types of DNA-BDs of mammalian transcriptional factors have been described: zinc finger motif in Sp1 (Kadonaga et al., 1987); homeodomain (HD) motif found in the octamer binding factors OCT-1, OCT-2, and the pituitary-specific factor Pit-1 (GHF-1) (Clerc et al., 1988; Ingraham et al., 1988; Ko et al., 1988; Muller et al., 1988; Sturm et al., 1988); and leucine zipper motif in C/EBP, Jun, and Fos (Kouzarides and Ziff, 1988; Landschulz et al., 1988a,b; Turner and Tjian, 1989). Deletion analysis has revealed that the DNA-BD of BTas is localized to the N-terminal of the protein. The DNA-BD region of BTas could form an α-helical structure consistent with a DNA-binding structure, but it does not have characteristic features of binding domains associated with zinc fingers, HDs, or leucine zippers. The primary sequences of several other recently cloned mammalian transcription factors indicate that there are other types of DNA-BDs in addition to the three described previously. For example, AP-2 (Williams et al., 1988) and the serum-response factor (SRF) (Norman et al., 1988) do not exhibit obvious similarities to the above-mentioned binding domains or among themselves.

Transcriptional activation domains can be divided into three key classes on the basis of their amino acid sequences: the acidic motif found in GAL4, GCN5, VP16, and p53 (He et al., 1993b; Van Hoy et al., 1993); glutamine-rich motif of Sp1 (Courey and Tjian, 1988); and proline-rich motif of CTF/NF-1 (Mermod et al., 1989). Similar to Bel1

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**Fig. 6.** Identify the minimal activation domain of BTas. (A) Schematic representation of BTas and deletion mutants. (B) A series of pCMV-BD-BTas deletions were transfected into 293T cells along with pFR-luc reporter gene and pCMV-β-gal as indicated. Forty-eight hours after transfection, luciferase activities were measured as described in Materials and methods. (C) β-Galactosidase activity in yeast cells transformed with the pGBKTT7, pGBKTT7-BTas (1–183 aa), pGBKTT7-BTas (198–249 aa), or pGBKTT7-BTas respectively. All transfections were performed in triplicate.
and Taf (Blair et al., 1994; Mergia et al., 1993), the activation domain of BTas is a potent activator when fused to the GAL4 DNA-BD. Therefore, BFV BTas, at least in this aspect, falls into the class of acidic transcriptional transactivators. BTas do not show any strong amino acid sequence homology to other acidic transcriptional transactivators, except for that one-fourth of the last 52 amino acids of BTas are acidic. In addition, the activation domain of BTas contains a high percentage of hydrophobic amino acids (34.6%). It was recently reported that the mutations of specific hydrophobic residues within the activation domain of herpes simplex virus (HSV) 1 VP16 and the Epstein–Barr virus Rta severely impaired the transactivation ability (Cress and Triezenberg, 1991; Hardwick et al., 1992; Ingles et al., 1991; Regier et al., 1993), suggesting that either specific hydrophobic interaction with protein partners or intramolecular folding is important for transactivation. It remains to be determined whether the activation domain of BTas functions via a novel mechanism involving both hydrophobic residues and acidic residues.

Sequence comparisons between the BFV BTas protein and the equivalent transactivators encoded by PVF, SFV-1, and SFV-3 reveal little level of conservation (Flugel et al., 1987; Mergia et al., 1991; Renne et al., 1992). A 15-aa conserved sequence motif is located in the activation domain of the three proteins Bel1, Taf1, and Taf3 (He et al., 1994), but this motif is not found in BTas. Different from Bel1, the deletion of 15 amino acids at the C-terminal severely diminished the activation ability of BTas. In contrast, the region from residues 90–127 appears to overcome the inhibitory effect. These results indicate that BTas contains at least one positive and one negative regulatory domain that modulate the distinct activation domain of BTas. The similar negative domain was also found in Bel1 (Lee et al., 1994).

The expression of BFV genes, like that of PVF, is regulated by the interplay of two viral promoters. In both the LTR and IP of BFV, the target sequence for BTas has been mapped to the LTR U3 region (–380/–140) (Liu et al., 2000) which is a typical inducible enhancer and IP (9205/9276) (Zhang et al., 2000). Although this finding strongly suggests that BTas acts via a DNA element, the precise sequence requirements for BTas function and whether BTas interacts directly or indirectly with its target sites have remained unclear. Herein, we performed a detailed analysis of the BTas responsive element in the LTR and IP of BFV. EMSA experiments revealed that BTas alone can directly bind to the TRE. In addition, sequence alignment and EMSA revealed four elements (–368/–346, –327/–307, –306/–285, and –186/–165) in the BFV LTR and one element (9243/9264) in the BFV IP. Unlike PVF, SFV-1, and SFV-3, the BFV TREs exhibit sequence homologies, and mutation of the conserved bases “TTA” in one of the TREs leads to the loss of binding ability of BTas. However, to our knowledge, the sequence does not show obvious homologies to known eukaryotic enhancer sequence motifs.

In this paper, we showed that the biological function domains of BTas involve an important DNA-BD and an active transcription activation domain. Further, we identified the five TREs of the BTas that binds to the LTR and IP. BTas might directly bind to the TREs in the LTR and IP through the N-terminal binding domain, whereas the C-terminal activation domain may recruit cellular cofactors required for BTas-mediated activation of transcription.

Materials and methods

Cloning of the plasmids

BFV pCMV-BTas and pLTR-luc were constructed as described previously (Liu et al., 1999). The pGEX-6p-1-BTas (1–133 aa), pGEX-6p-1-BTas (1–217 aa), pGEX-6p-1-BTas (27–249 aa), and pGEX-6p-1-BTas were constructed by inserting individual PCR fragment into the pGEX-6p-1 (Amerham Pharcmaica). The pCMV-AD-BTas (27–249 aa), pCMV-AD-BTas (1–133 aa), pCMV-BD-BTas (90–249 aa), pCMV-BD-BTas (126–249 aa), pCMV-BD-BTas (167–249 aa), pCMV-BD-BTas (198–249 aa), pCMV-BD-BTas (167–234 aa), pCMV-BD-BTas (198–234 aa), pCMV-BD-BTas (1–184 aa), and pCMV-BD-BTas were constructed by inserting individual PCR fragment into the pCMV-BD or pCMV-AD vector (Invitrogen). The pGBK7-BTas (1–183 aa), pGBK7-BTas (198–249 aa) and pGBK7-BTas were constructed by inserting individual PCR fragment into the pGBK7 vector (Clontech). All the new constructs were confirmed by sequencing.

Cell culture, transfection, and luciferase reporter assays

293T cells were grown in Dulbecco’s modified Eagle medium (DMEM) (GIBCO, Grand Island, NY) supplemented with 10% fetal bovine serum (FBS) and antibiotics. 293T cells were seeded at 1×10⁵ cells per well in 12-well plates. The following day, cells were transfected with pFR-luc and a series of pCMV-BD-BTas deletions, with pCMV β-gal as the control of transfection efficiency using polyethylenamines (PEI) according to the manufacturer’s protocol (Durocher et al., 2002). Total amount of DNA were equilibrated by adding parent plasmids. At 48 h after transfection, the cells were harvested and luciferase assays (Promega) were performed. The luciferase activities were normalized by β-galactosidase activities. All transfections were performed in triplicate.

Yeast transactivation studies

The pGBK7, pGBK7-BTas (1–183 aa), pGBK7-BTas (198–249 aa), or pGBK7-BTas plasmids were transformed into the yeast strain AH109. After three days of growth selection on SD-Trp plates at 30 °C, at least three colonies isolated from plates were grown in liquid culture and assayed for β-galactosidase (β-Gal) activity by standard protocols.

Electrophoretic mobility shift assay (EMSA)

GST fusion proteins were expressed in Escherichia coli strain BL21 and subsequently purified by glutathione sepharose beads (Amerham Biosciences) with PreScission Protease according to the manufacturer’s instruction. Protein concentrations were determined by Bradford assay. The binding reaction was carried out with 500 ng of purified protein.

The BFV LTR probes (–380/–140, –380/–277, –310/–140, –239/–140, –200/–140, and –239/–170) used for EMSA were generated by PCR, other probes (–368/–346, –327/–307, –310/–277, –310/–257, –306/–285, –186/–165, –161/–140, and 9243/9264) were synthesized and annealed. All probes were labeled by digoxin using the DIG Gel Shift Kit (Roche). For competition experiments, unlabeled competitor oligodeoxynucleotides were added in 20-fold molar excess at the preincubation period.

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