Evaluation of the $\delta$ subunit of bovine adaptor protein complex 3 as a receptor for bovine leukaemia virus

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A candidate gene of the bovine leukaemia virus (BLV) receptor (BLVR) was cloned previously and predicted to encode a transmembrane protein. Subsequent cloning of related genes from other organisms indicated that the candidate gene is related, but unique, to a gene family of the $\delta$ subunit of the adaptor protein (AP) complex 3, AP-3. Therefore, bovine cDNAs (boAP3$\delta$) that are highly homologous to the candidate gene were cloned and sequenced. The nucleotide sequences suggested that the boAP3$\delta$ cDNA encodes the $\delta$ subunit of boAP3 without transmembrane domains. Part of the AP3$\delta$ cDNA isolated from the lymph node, spleen and MDBK cells, from which the BLVR candidate cDNA was derived, has almost the same nucleotide sequences as the boAP3$\delta$ cDNA. A boAP3$\delta$ protein tagged with green fluorescent protein was localized in the cytoplasm and incorporated into AP-3 in bovine cells. Unlike the previous report about the candidate gene, the boAP3$\delta$ gene introduced into murine NIH 3T3 cells did not increase the susceptibility of the cells to BLV infection. Many small insertions and deletions of nucleotides could generate the predicted transmembrane and cytoplasmic regions of the BLVR protein from the prototypic boAP3$\delta$ gene.

INTRODUCTION

Many retroviruses bind to specific receptors on the target cell surface to initiate infection and the receptors are one of the important factors determining the host range of retroviruses. Many receptors for retroviruses have been identified that are either multiple or single membrane-spanning proteins (Sommerfelt, 1999). BLVRcp1, a cDNA encoding the candidate protein of the bovine leukaemia virus (BLV) receptor (BLVR), was cloned from an expression library of the bovine cell line MDBK, susceptible to BLV infection based on the binding property of bacterially expressed protein to BLV gp51 (Ban et al., 1993). According to the nucleotide sequence, the candidate protein was predicted to be a type I transmembrane protein with a single transmembrane domain and transfection of a BLVRcp1 expression plasmid into mouse and human cells increased the susceptibility of cells to recombinant BLV infection (Ban et al., 1993).

We cloned previously a mouse cDNA (mBLVR1) homologous to bovine BLVRcp1 (Ban et al., 1993) and BLVRcp1/5′, missing the 5′-part of BLVRcp1 (Ban et al., 1994) (BLVRcp) based on cross-hybridization, and found that mBLVR1 encodes a protein without the typical hydrophobic transmembrane region and is closely related to the $\delta$ subunit of the adaptor protein (AP) complex 3, AP-3 (Suzuki & Ikeda, 1998). In humans and mice, there are four different AP complexes, AP-1, -2, -3 and -4, and all of them mediate intracellular protein transport (Boehm & Bonifacino, 2002; Robinson & Bonifacino, 2001). The AP complexes consist of four different subunit proteins, each of which belongs to the $\gamma$/$\delta$/$\alpha$, $\beta$, $\mu$ and $\sigma$ gene families, respectively, and AP-3 has the $\delta$, $\beta$, $\mu$ and $\sigma$ subunits (Boehm & Bonifacino, 2002; Robinson & Bonifacino, 2001). Humans and mice appear to carry only one $\delta$ gene expressed ubiquitously (Boehm & Bonifacino, 2001). Although BLVRcp is clearly related to the $\gamma$/$\delta$/$\alpha$ subunit family at the nucleotide sequence level, the predicted protein structure is unique in the family because none of the other members has any transmembrane domains. To address the questions of whether BLVRcp is a representative of the bovine AP3$\delta$ homologue (boAP3$\delta$) and if not, how BLVRcp was different from the AP3$\delta$ family, we recloned bovine BLVRcp-related cDNAs from the brain, lymph node and spleen and from MDBK cells, from which BLVRcp1 was isolated originally. We then characterized their encoding proteins for their potential to interact with other boAP3 subunits. We also tested the susceptibility of the cells transfected with the cloned cDNA to BLV infection.
METHODS

Cells and viruses. BT cells (ATCC, #CRL1390) (McClurkin et al., 1974) were derived from bovine turbinate; MDBK cells (ATCC, #CCL-22) were derived from bovine kidney; NIH 3T3 cells (ATCC, #CCL-92) were derived from mouse embryo; CC81 cells were derived from a cat (Fischinger et al., 1974) and used for BLV titration. The bat BLV-Bat-c12 cells produce high amounts of infectious BLV (Graves & Ferrer, 1976). All cells were cultured with Dulbecco’s modified Eagle’s medium (DMEM) (Nissui) containing 0.25 % HEPES, 50 μg kanamycin ml−1 and 5–10 % foetal calf serum. The BLV-Bat-c12 cells were stably transfected with plasmid pBLV-SVNEO, encoding a defective BLV with a neomycin-resistant gene (Deren & Martarano, 1990) (a gift from D. Deren, National Cancer Institute–Frederick Cancer Research Facility, Maryland, USA) and the culture supernatant was used as infectious virus (BLV-neo).

Cloning of boAP3δ cDNAs. A λ phage library of bovine brain cDNA (IZAP II vector, a gift from M. Sakurai, National Institute of Agrobiological Sciences, Japan) was screened with a 32P-labelled probe of the 1 kb EcoRI–HindIII fragment, nt 5–996, derived from the bovine BLV/pcp1 clone (Ban et al., 1993) (a gift from R. Kettmann, Faculty of Agronomy, Belgium), as described previously (Suzuki & Ikeda, 1998). The brain cDNA library was constructed for other purposes (Kubota et al., 1994). To isolate longer cDNA clones, the DNAs of 90 independent pools of phages obtained from a single area of about 5–10 plaques in the first screening were analysed for the size of a 5’ region of cDNAs by PCR using primers of mBLV1 (mo-9209R, 5’-ATGGAGGCCCATGTTGTCG-3’) and vector (M13-20, 5’-GTAAAACGACGGCCAGT-3’). PCR products were subjected to electrophoresis through agarose gel followed by Southern blot hybridization. The labelled cell lysates (3 × 10⁵ c.p.m. per lane) were incubated with protein G–Sepharose (Amersham Pharmacia) for 1 h at 4˚C. After washing five times with TNE buffer (10 mM Tris/ HCl pH 7.8, 1% NP-40, 0.1 M NaCl and 1 mM EDTA) containing the protease inhibitor cocktail (Complete; Roche) at 4˚C. The labelled cell lysates (3 × 10⁵ c.p.m. per lane) were incubated with protein G–Sepharose (control) or protein G–Sepharose preabsorbed with 5 μg of the anti-GFP antibody (clone 3E6; Wako) or anti-MHC class I antibody (clone IL-A88; a gift from J. Naessens, International Livestock Research Institute, Kenya) (Toye et al., 1990) for 1 h at 4˚C. After washing with TNE, the precipitates were boiled in sample loading buffer (130 mM Tris/HCl pH 6.8, 6% SDS, 20% glycerol, 10% 2-mercaptoethanol and 0.005% bromphenol blue) and fractionated by SDS-PAGE on a 7% gel. Isotope signals were detected using a bio-imaging analyser (BAS 2000; FujiFilm) by exposing an imaging plate for 5 days.

Immunoprecipitation. BT cells and BT cells stably transfected with the pCMV-GFP/δ expression plasmid (BT-GFP/δ) were metabolically labelled with [35S]methionine and [35S]cysteine (Express Protein Labelling mix; NEN Life Science) for 7 h. After washing with cold medium (DMEM), the cells were lysed with TNE buffer (10 mM Tris/HCl pH 7–8, 1% NP-40, 0.15 M NaCl and 1 mM EDTA) containing the protease inhibitor cocktail (Complete; Roche) at 4˚C. The labelled cell lysates (3 × 10⁵ c.p.m. per lane) were incubated with protein G–Sepharose (control) or protein G–Sepharose preabsorbed with 5 μg of the anti-GFP antibody (clone 3E6; Wako) or anti-MHC class I antibody (clone IL-A88; a gift from J. Naessens, International Livestock Research Institute, Kenya) (Toye et al., 1990) for 1 h at 4˚C. After washing five times with TNE, the precipitates were boiled in sample loading buffer (130 mM Tris/HCl pH 6–8, 6% SDS, 20% glycerol, 10% 2-mercaptoethanol and 0.005% bromphenol blue) and fractionated by SDS-PAGE on a 7% gel. Isotope signals were detected using a bio-imaging analyser (BAS 2000; FujiFilm) by exposing an imaging plate for 5 days.

Fluorescence microscopy. After transfection of BT cells with pCMV-GFP or pCMV-GFP/δ and pSV2-hph, and selection of the transformed cells with medium containing 100 μg hygromycin B ml−1, the resulting 10–20 hygromycin-resistant colonies were mixed and passaged several times. The mixed cell populations were analysed for expression of GFP fluorescence by fluorescence microscopy. BT-GFP/δ and BT-GFP cells, BT cells transfected with pCMV-GFP, were plated on an 8-well chamber slide at a density of 1 × 10⁵ cells per well. After 14 h, the cells were washed with cold PBS and fixed with PBS containing 4% paraformaldehyde for 30 min at 4˚C. After washing with cold PBS, the slide was mounted with Aqua Poly/Mount reagent (Polysciences). The cells were observed by fluorescence microscopy and differential interference microscopy using a LEITZ DMRD microscope (Leica) and a digital CCD camera (Hamamatsu Photronics).

Immunoblotting. BT and BT-GFP/δ cells were lysed with digitonin buffer (1% digitonin, 10 mM triethanolamine/HCl pH 7–8, 150 mM NaCl, 10 mM iodocetamide and 1 mM EDTA) containing the protease inhibitor cocktail at 4˚C. Cell lysates (2.5 × 10⁶ cells per lane) were preabsorbed with protein G–Sepharose 4 Fast Flow and a control mouse antibody (anti-His antibody; Amersham Pharmacia), which was the same isotype as the anti-GFP antibody, and then immunoprecipitated with the mouse anti-GFP antibody (clone 3E6), as described above. Immunoprecipitates were boiled in sample loading buffer (187.5 mM Tris/HCl pH 6–8, 6% SDS, 30% glycerol and 0–03% phenol red), fractionated by SDS-PAGE on a 6–5% or a 12% gel and transferred onto a PVDF membrane (Immobilon;
Millipore). The membranes were incubated in PBS containing 5% ECL blocking agent (Amersham Pharmacia) and 0-05% Tween 20, and then probed with either the rabbit anti-Δ or anti-α subunit antibodies (Simpson et al., 1997) (gifts from S. M. Robinson) followed by treatment with a horseradish peroxidase (HRP)-conjugated anti-rabbit IgG antibody (Zymed). The HRP-mediated chemiluminescent reaction was performed with ECL Plus Western Blotting Detection reagents (Amersham Pharmacia). The same membranes were reprobed with rabbit anti-β or anti-μ subunit antibodies (Simpson et al., 1996) (gifts from S. M. Robinson) after stripping of the first probed antibody with Restore Western Blot Stripping buffer (Pierce).

Infection of cells with recombinant BLV. NIH 3T3 cells were plated at a density of 1-2 × 10^5 cells per 10 cm diameter dish and on the following day transfected with pCMV-Δ, pCMV-BLVR or pCMV-GFPΔ. After 14 h, the cells were trypsinized and replated on a new 10 cm dish at a density of 5 × 10^5 cells per dish. At the same time, other cells were also plated at the same cell density. After 9 h, polybrene (Nacalai Tesque) was added to the culture medium at a final concentration of 20 μg ml^-1 and the cells were incubated for a further 1 h. The cells were then infected with BLV-neo (Derse & Martarano, 1990) with polybrene for 1 h. After a 2 day culture, the medium was replaced with a culture medium containing 0-8 mg genetecin ml^-1 (G418 sulfate) (Sigma) and the developed G418-resistant colonies were counted after 10–16 days of culture. Expressions of transfected genes at the time of the BLV-neo infection were verified by RT-PCR.

RESULTS

Cloning of boAP3Δ cDNA

We screened a cDNA library derived from bovine brain (Kubota et al., 1994) using a probe from a portion of BLVRcp (Ban et al., 1993). The two largest cDNAs were cloned into plasmids and sequenced completely. They were 4683 and 4256 bp in size and tentatively termed boAP3 and boAP3Δ. They were many characteristics with human AP3Δ (hAP3Δ) (Ooi et al., 1997; Simpson et al., 1997) and the murine BLVR homologue protein (mBLVR) (Suzuki & Ikeda, 1998). The positions of the first ATG codon at nt 32 and the termination codon at nt 3652 were equivalent to those of hAP3Δ and mBLVR1 (Fig. 1). The encoded protein showed an 81-8% (Simpson et al., 1997) or 86-7% (Ooi et al., 1997) identity with the two hAP3Δ clones and 88-3% identity with mBLVR (Suzuki & Ikeda, 1998). A hydrophobic profile of the encoded protein also resembled those of hAP3Δ and mBLVR (data not shown). Therefore, the cloned cDNAs appeared to encode a bovine homologue of the Δ subunit of AP-3.

The boAP3Δ1 cDNA has almost the same size as the AP3Δ mRNAs of humans (Ooi et al., 1997; Simpson et al., 1997) and mice (Suzuki & Ikeda, 1998), but BLVRcp (Ban et al., 1993, 1994) was 1-5 kb shorter than boAP3Δ1 (Fig. 1). The nucleotide sequences of boAP3Δ1 and BLVRcp show a very high identity throughout the overlapping region: 99-6% in the protein-encoding region and 95-7% in the 3′ noncoding region of boAP3Δ1 (Fig. 1), but their deduced protein structures are quite different. boAP3Δ1 has a termination codon at nt 3653, which is located upstream of the transmembrane domain of BLVRcp and does not seem to encode a transmembrane protein (Figs 1 and 2). The overlapping region of the two protein sequences shows a high identity (99-6%), except for a block from aa 906 to 964, in which the two proteins have the same number of amino acids but no significant identities (Fig. 2A). The nucleotide sequences of this region are identical, except for each of the ends of the region: BLVRcp has a one base deletion at nt 2745 and a one base insertion at nt 2922 of boAP3Δ1 (Fig. 2B). This lack of amino acid identity should be due to frameshifts caused by the insertion and deletion. The TGA sequence at nt 3653 of boAP3Δ1 is used as a stop codon, while this sequence is used for amino acids in BLVRcp (Fig. 2B). This difference is probably due to a frameshift by a one base insertion in BLVRcp at nt 3641 of boAP3Δ1, 13 bases upstream of the boAP3Δ1 stop codon. The open reading frame (ORF) of BLVRcp extends a further 836 bases to the 3′ terminus but the corresponding region of boAP3Δ1 includes 21 stop codons in the three frames. These stop codons were also used as amino acids because of the 12 one base, five two base and one four base insertions or deletions in BLVRcp.

Fig. 1. Schematic representation of boAP3Δ1, BLVRcp, mBLVR1 and hAP3Δ cDNAs. Grey boxes show ORFs of the cDNAs. Initiation codons (Met) of each clone are shown by arrows. The hatched box shows the predicted transmembrane (TM) domain of BLVRcp (Ban et al., 1993). Numbers are expressed as nucleotide positions relative to boAP3Δ1. Nucleotide identities (%) between boAP3Δ1 and BLVRcp are also shown.
We amplified about 2 kb of the cDNAs (nt 2351–4298 of boAP3δ1) by RT-PCR from the bovine lymph node and spleen and MDBK cells using primers that are accordant with boAP3δ1 and BLVRcp1. A 1-8 kb region (nt 2390–4198), including the insertions and a deletion causing the lack of amino acid identity and skipping of the stop codon in BLVRcp (Fig. 2), were sequenced directly. Between boAP3δ1 and MDBK cDNA, only one nucleotide variation was observed at nt 3291 and this does not change the amino acid sequence. The cDNA sequences of the spleen and lymph node derived from the same animal were a mixture of both types. The expression of BLVRcp-type mRNA-carrying transmembrane region was not detected in MDBK cells.

Expression and localization of the GFP-boAP3δ fusion protein

All of the reported AP complexes are localized in the cytoplasm and mediate protein transport (Boehm & Bonifacino, 2002; Robinson & Bonifacino, 2001). AP-3 is associated at the trans-Golgi network in the cytoplasm (Dell’Angelica et al., 1997; Simpson et al., 1997). However, some intracellular proteins, such as heat shock proteins, are expressed occasionally on the cell surface (Multhoff & Hightower, 1996). Therefore, we established a BT-GFP/δ cell line expressing boAP3δ tagged with GFP and investigated the cellular localization of the protein.

The expression of GFP/δ was verified by immunoprecipitation of BT-GFP/δ cells (Fig. 3) and flow cytometry (data not shown). Lysates of BT and BT-GFP/δ cells metabolically labelled with [35S]methionine and [35S]cysteine were immunoprecipitated with the anti-GFP antibody. As a control, the anti-bovine MHC class I antibody was used because MHC class I molecules are expressed in a wide range of cells. The anti-MHC class I antibody precipitated proteins of about 190 and 100 kDa, in addition to a minor 50 kDa protein only from BT-GFP/δ cells but not from BT cells (Fig. 3, lane 6). A Western blot analysis of an unlabelled BT-GFP/δ cell lysate also detected an identical major 190 kDa band in addition to several bands ranging from 70 to 160 kDa with the anti-δ subunit antibody (see Fig. 5, lane 2). The molecular mass of GFP/δ calculated by the amino acid content was 163 kDa from the sum of the 136 kDa boAP3δ and the 27 kDa GFP but hAP3δ showed an apparent molecular mass of 160 kDa, despite its calculated molecular mass being between 125 and 130 kDa (Ooi et al., 1997; Simpson et al., 1997). Therefore, we speculate that the 190 kDa band would be an entire GFP/δ protein and the other smaller bands might be immature or degradation products of GFP/δ.

Cellular localization of GFP/δ in BT-GFP/δ cells was observed using a fluorescence microscope (Fig. 4). Granular fluorescence was observed in the cytoplasm but not on the cell surface (Fig. 4C). This pattern of localization was similar to that of endogenous AP-3 in bovine MDBK cells (Simpson et al., 1997). On the other hand, in BT-GFP cells expressing GFP alone, green fluorescence was located preferentially in the nucleus and diffusely in the cytoplasm, as expected (Fig. 4A).

Inclusion of GFP-boAP3δ within AP-3

To investigate whether the protein encoded by the boAP3δ1 cDNA is the bovine AP3δ itself, we tested the possible incorporation of the constructed GFP/δ protein into the AP-3 of bovine cells. Lysates of BT and BT-GFP/δ cells were prepared with digitonin buffer, which generally
leaves protein complexes intact, and were immunoprecipitated with the mouse anti-GFP antibody. The precipitated protein complexes were washed extensively, electrophoresed under nonreducing conditions and blotted onto membranes. The membranes were then probed with each of the rabbit polyclonal antibodies to the δ, β3, μ3 or σ3 subunits of the human or rat AP-3 (Simpson et al., 1996, 1997). These antibodies reacted with the subunits of AP-3 but did not cross react with the components of AP-1, such as γ, β1, μ1 and σ1 (Simpson et al., 1997). The anti-δ antibody detected a 190 kDa protein and several proteins ranging from 70 to 160 kDa (Fig. 5, lane 2). The anti-β3, -μ3 and -σ3 antibodies reacted with a protein of approximately 140 kDa (Fig. 5, lane 4), a 45 kDa protein (lane 6) and a 20–23 kDa protein (lane 8), respectively, all of which were the equivalent sizes of the human proteins (Ooi et al., 1997). A Western blot experiment after fractionation under reducing conditions showed the same pattern of protein bands as the experiment under nonreducing conditions (data not shown). Strong signals of about 30 kDa shown on the blots with anti-μ3 and anti-σ3 antibodies (Fig. 5, lanes 5–8) were also reactive with the HRP-labelled anti-mouse IgG antibody (data not shown), indicating a cross reaction of the mouse anti-GFP antibody used for the first immunoprecipitation to the HRP-labelled anti-rabbit IgG antibody used for immunoblots. Thus, coprecipitation of GFP/δ with the other subunits of AP-3 indicated the inclusion of GFP/δ in bovine AP-3.

**Effect of boAP3δ expression on susceptibility of cells to BLV infection**

As the transfection of mouse NIH 3T3 and human Hep-2 cells with the bovine BLVRcp1 increased the susceptibility of cells to infection by the recombinant pseudotype BLV carrying the lacZ gene by about 3- to 100-fold (Ban et al., 1993), we evaluated the effects of our boAP3δ upon BLV infection. Because, despite our repeated attempts, we could not obtain a stable transformant of NIH 3T3 cells expressing boAP3δ, we performed transient transfections of NIH 3T3 cells with pCMV-δ, pCMV-BLVR or pCMV-GFP/δ and infections with a recombinant BLV-neo pseudotype virus carrying a neomycin-resistant gene (Derse & Martarano, 1990). G418-resistant colonies were counted 10–16 days later (Table 1). Neither of the NIH 3T3-δ cells expressing boAP3δ and NIH 3T3-GFP/δ cells expressing GFP/δ showed increased susceptibility to BLV infection compared to the parental NIH 3T3 cells. In spite of a previous report, the expression of BLVRcp in NIH 3T3 cells had no effect on the susceptibility of the cells to BLV infection. BLV is known to infect cells of various animal species. In our infectivity assay, two bovine cells (MDBK and BT) and CC81 cells were highly susceptible, whereas the BLV-Bat2cl1 cells persistently infected with BLV produced very few colonies, probably via a receptor interference mechanism (Table 1).
**DISCUSSION**

We cloned the almost full-length cDNA of the bovine boAP3δ gene, whose nucleotide sequences are collinearly aligned with that of the bovine cDNA BLVRcp of the BLVR candidate gene (Ban et al., 1993, 1994). It is apparent that the boAP3δ cDNAs were derived from a bovine AP3δ gene or its closely related gene because of high amino acid identities with the human (Ooi et al., 1997; Simpson et al., 1997) and probably mouse (Suzuki & Ikeda, 1998) AP3δ genes. In addition, GFP/δ was incorporated into AP-3 in bovine cells (Fig. 5). We also isolated the three cDNAs of the 3′-half of the boAP3δ gene from bovine lymph node and spleen, which are target organs of BLV infection, and MDBK cells from which the candidate gene was cloned originally. Their nucleotide sequences were almost identical with that of the boAP3δ cDNA but clearly differed from that of the BLVR candidate gene.

The major difference between boAP3δ and BLVRcp is their predicted proteins. The BLVR candidate gene encodes a protein with a transmembrane domain (Ban et al., 1993, 1994), whereas boAP3δ encodes a protein with no obvious hydrophobic region, as in the case of hAP3δ and its probable mouse homologue (Ooi et al., 1997; Simpson et al., 1997; Suzuki & Ikeda, 1998). Little is known about the physiological function, biochemical properties or cellular localization of the BLVR candidate gene product. When the nucleotide sequences are compared, the identities are high in both the protein-encoding region (99-67%) and the 3′ noncoding region (95-7%) of boAP3δ. No large sequence gap is observed. Instead, many small insertions and deletions scattered at various positions could cause the several crucial differences in the proteins. First, the portion (aa 906–964) of boAP3δ representing about 10% of the overlapping region lacks identity with BLVRcp. This appears to be due to the one base insertion and the one base deletion (Fig. 2A, B). Second, the stop codon of the boAP3δ ORF appears to be skipped in BLVRcp by a frameshift caused by one base insertion at the position 13 bases upstream from the stop codon (Fig. 2B). Lastly, the BLVRcp ORF extends 836 bp from the 3′ end of the boAP3δ ORF. This can be explained also by many small insertions and deletions leading to skips of the many stop codons lying in the boAP3δ 3′ noncoding region (Fig. 2B).

The origin of the BLVRcp cDNA is unknown, although the close relationship between BLVRcp and AP3δ is indicated clearly by the nucleotide sequence identities even in the noncoding region, as described already. A few possibilities can be considered, such as cloning artefacts, the allelic variant or an unidentified AP3δ-related gene. Cloning artefacts are probable because no other gene encoding the BLVRcp-like protein has ever been identified, either by others or by us. The three BLVRcp cDNA clones in the original study (Ban et al., 1993) might be amplified from one clone because they have identical inserts. A variant or mutant allele at the bovine AP3δ locus is possible and it might be unique to the MDBK cells or BLV-permissive cells in the animal. However, we could not detect BLVRcp-like cDNA in the MDBK cells, lymph node or spleen even if we used PCR primers that should amplify both cDNAs. The existence of an unidentified AP3δ-related gene cannot be ruled out. The adaptor subunit gene family and the related gene family are thought to be derived from a common ancestral gene (Boehm & Bonifacino, 2001; Schledzewski et al., 1999). Considerable variations have been found within these gene families, such as naturally occurring mutations, pseudogenes, alternatively spliced mRNAs and isoforms encoded by distinct genes (Boehm & Bonifacino, 2001). In the AP3δ gene family, the deletion mutant gene moiça was found in mice (Kanethi et al., 1998) and two AP3δ cDNAs with an internal deletion or insertion were reported in humans (Ooi et al., 1997; Simpson et al., 1997). However, our previous Southern blot hybridization of bovine DNA with a BLVRcp probe did not positively support the existence of an additional AP3δ-related locus in the bovine genome (Suzuki & Ikeda, 1998). Nevertheless, the cloning and analysis of the respective chromosomal genes should clarify this point.

We could not establish any stable transformant of AP3δ-expressing NIH 3T3 cells, although no obvious cell damage was observed several days after transfection if the cells were cultured without antibiotic selection; the reason for this is unknown. In our transient transfection experiments, neither the AP3δ cDNA nor the BLVRcp cDNA conferred susceptibility to the BLV-neo virus in NIH 3T3 cells, in
contrast to the successful induction of BLV-susceptibility in stable and transient transformants of the NIH 3T3 cells via the introduction of a BLVRcp expression vector (Ban et al., 1993). We do not know the reason for this discrepancy. Differences in the many experimental materials and methods, such as expression vectors, length and sequence of the ORFs inserted into vectors and infected viruses, are noted. More studies are required to reevaluate the significance and generalization of the BLV receptor candidate gene.

ACKNOWLEDGEMENTS

We thank Dr Richard Kettmann for providing the plasmid BLVRcp1, Dr David Derse for the plasmid pBLV-SVNeo, Dr Michiharu Sakurai for the bovine cDNA library, Dr Jan Naessens for the monoclonal antibody clone IL-A88 and Dr Margaret S. Robinson for the antibodies against AP-3 subunits. We thank Dr Shozo Arai and Dr Manabu Yamada for their technical support. This study was supported in part by grants from the Ministry of Agriculture, Forestry and Fisheries of Japan.

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