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Molecular Cloning of Biologically Active Proviruses of Bovine Immunodeficiency-like Virus[1]

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A series of independent proviral molecular clones of bovine immunodeficiency-like virus (BIV) obtained from a genomic library of BIV-infected bovine cell DNA were physically and biologically characterized. Heteroduplex mapping shows that two of these BIV clones (106 and 127) contain uninterrupted proviral sequences approximately 9.0 kb in length, flanked by nonhomologous bovine cellular sequences. Microinjection of purified DNA from BIV clone 106 or 127 into susceptible bovine cells produces virus-specific cytopathic effects, including syncytium induction, supernatant reverse transcriptase activity, and infectious virus particle formation, similar to the effects produced by parental virus stock. Using restriction enzyme mapping, it was determined that the two infectious clones share 13 of 14 sites mapped within the provirus; thus, based on this criterion, the two clones are nearly identical, with the exception of a single polymorphic site recognized in the 3′ half of the genome. BIV appears to be an exogenous pathogenic virus, because Southern hybridization analyses detected no endogenous sequences related to BIV in DNA from a variety of uninfected bovine cells and tissues. Most of the BIV-related DNA found in cells 96 hr after infection is present as linear unintegrated viral DNA, although the presence of host flanking sequences in our proviral clones indicates that integration takes place. These biologically active clones of BIV will be of use in defining further the mechanisms of BIV pathogenesis and in engineering specific diagnostic reagents to determine the prevalence of BIV in cattle populations.

INTRODUCTION

The Lentivirinae subfamily of retroviruses is a group of exogenous, nononcogenic viruses that cause chronic, multisystem diseases in susceptible hosts. Lentiviruses have been causally associated with an array of pathological syndromes that commonly include clinical manifestations such as fever, anemia, central nervous system lesions, pneumonia, lymphadenopathy, immunodeficiency, and generalized wasting (Haase, 1986; Gonda, 1988). Symptoms are usually slow in onset but chronic and progressive. The disease progression is variable and may be accompanied by opportunistic infections, such that individual hosts may display a mosaic of pathology related to the virus infection.

While lentiviruses have received attention for some time as novel animal pathogens and putative models of human disease, their study has taken on new importance with the discovery that the human immunodeficiency virus (HIV-1), the causative agent of the acquired immunodeficiency syndrome (AIDS), is a lentivirus (Gonda *et al.*, 1985, 1986; Sonigo *et al.*, 1985; Chiu *et al.*, 1985; Stephens *et al.*, 1986). This phylogenetic relationship suggests that the similarities between animal lentivirus diseases and AIDS (Gonda *et al.*, 1986; Haase, 1986; Gonda, 1988) have a genetic basis and that information gained from research on lentiviruses may prove useful in understanding the pathogenesis of HIV.

An infectious, pathogenic viral agent capable of inducing syncytia in cell culture and having an ultrastructural morphology most similar to that of a lentivirus was isolated from cattle by Van Der Maaten *et al.* (1972). This bovine virus causes lymphadenopathy, lymphocytosis, central nervous system lesions, progressive weakness, and emaciation. Recently, we have shown that this agent encodes a reverse transcriptase with Mg^{2+} cation preference; has immunological cross-reactivity with HIV-1, simian immunodeficiency virus, and equine infectious anemia virus; and, in terms of DNA sequence homology in the highly conserved reverse transcriptase domain of the *pol* gene, is much closer to the lentiviruses than to any other retrovirus subfamily (Gonda *et al.*, 1987). These studies conclusively demonstrated that this agent, which we call bovine immu-

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nodeficiency-like virus (BIV), is a lentivirus. To further characterize BIV and to provide fundamental reagents for detailed molecular, pathogenetic, and epidemiologic studies, we have derived biologically active proviral molecular clones of BIV. Here we report on the isolation, characterization, and biological function of these clones.

MATERIALS AND METHODS

Virus and cell culture

The parental stock of BIV was previously described (Van Der Maaten *et al.,* 1972). Bovine epithelial trachea (EBTr) cells used for the propagation and molecular cloning of BIV were obtained from the American Type Culture Collection (Rockville, MD). For microinjection experiments, we used primary cultures established from fetal cells of various tissues of a first trimester male bovine fetus, as previously described (Gonda *et al.,* 1987). Cells were grown in Dulbecco's modified Eagle's medium supplemented with 10–15% fetal calf serum, 1% penicillin/streptomycin, and 1% glutamine in a humidified atmosphere of 5% CO_2 at 37°. BIV is cytopathic for EBTr cells, the most notable effect being syncytium induction and cell death, and EBTr or other primary bovine cells infected with BIV do not form continuous cell lines and express virus for only a few passages; thus, it is difficult to study the long-term effects of BIV infection *in vitro.* To subculture and amplify the infection, and in order to obtain sufficient quantities of infected cell DNA for cloning experiments, we cocultured BIV-infected and uninfected EBTr cells at a ratio of 1:10 when maximum cytopathic effect was observed in infected cultures. The methods of virus propagation for the large-scale production and purification of virions were performed as described (manuscript in preparation).

Reverse transcriptase assay

Reverse transcriptase assays were performed as described (Gonda *et al.,* 1987) using protocols of Hoffman *et al.* (1985).

RNA and DNA isolation

Viral RNA was isolated as previously described (Gonda *et al.,* 1980) with minor modifications. Briefly, 1 ml of 1000× concentrated sucrose-gradient banded virus was brought to 1% final concentration in sodium dodecyl sulfate. Proteinase K was added to a final concentration of 0.5 mg/ml and incubated for 30 min at 50°. The solution was then extracted three times with equal volumes of buffer-saturated phenol, followed by two extractions with a mixture of chloroform and isoamyl alcohol (24:1). The aqueous phase was adjusted to 0.3 *M* sodium acetate and RNA was precipitated at −20° with 2 vol of cold ethanol. The viral RNA pellet was resuspended in 1 ml of 10 m*M* Tris buffer (pH 8.0), 0.1 m*M* EDTA (TLE). The extraction and precipitation steps were repeated and the final RNA pellet was resuspended in 100 μl of TLE. All aqueous solutions were made with glass distilled, deionized water treated with diethylpyrocarbonate and autoclaved before use to inhibit RNase activity.

Total genomic DNA was isolated from BIV-infected EBTr cells 96 hr after subculturing the infection. Six T-150 flasks containing confluent monolayers of BIV-infected cells were decanted of medium and washed gently once in 1× Dulbecco's phosphate-buffered saline. The wash was drained from the flask completely by inversion and 3 ml of lysing buffer (0.6% sodium dodecyl sulfate, 20 m*M* Tris buffer, pH 8.0, and 50 m*M* EDTA) was added. The flask was gently agitated to evenly distribute the liquid and to lyse the cells for 30 min at room temperature. The flask was placed on end for 5 min to concentrate the cell/DNA slurry and the liquid containing DNA was collected and twice extracted with equal volumes of buffer-saturated phenol/chloroform (1:1) followed by chloroform/isoamyl alcohol (24:1). DNA was ethanol precipitated by the addition of 2 vol of −20° absolute ethanol to the recovered supernatant, spooled on a Pasteur pipet, and washed extensively with cold 70% ethanol. Spooled DNA was allowed to partially dry under negative pressure and then was dissolved in 10 m*M* Tris (pH 8.0), 1 m*M* EDTA at 4–10° overnight. DNA from other uninfected and BIV-infected bovine cells was extracted similarly.

λ library preparation and screening

A representative genomic library in the bacteriophage λ vector EMBL3 (Frischauf *et al.,* 1983) was prepared from BIV-infected EBTr cell DNA after partial digestion with *Mbo*I and size selection (14- to 24-kb fragments) on sucrose density gradients (Maniatis *et al.,* 1982). This size range would have excluded packaging of unintegrated viral DNA. The library was packaged into infectious particles using Gigapack Gold packaging extracts according to the directions of the supplier (Stratagene). When titered on *Escherichia coli* strain KH802, the packaging efficiency of the library was 1.7×10^6 plaque-forming units (PFU) per microgram of ligated DNA. Library aliquots were plated at high density (60,000 PFU/150-mm dish) and screened by hybridization with radioactive cDNA probes made to viral RNA as described below (Benton and Davis, 1977; Maniatis *et al.,* 1982). Positive clones were plaque purified through two more rounds of hybridization and screen-

ing at successively lower plaque densities (10,000 and 500 PFU/150-mm dish). Replicate nitrocellulose filters were lifted and probed for each screen.

Radioactive probes

A representative cDNA probe was made from viral RNA using AMV reverse transcriptase (Bethesda Research Laboratories) as described by Mullins *et al.* (1980). Three hundred micrograms of uninfected cellular RNA per microgram of reverse-transcribed viral RNA was added as an unlabeled competitor to hybridizations in which cDNA probes were used. Probes specific for the *pol* genes of HIV, visna virus, and BIV were radiolabeled by nick-translation (Rigby *et al.*, 1977) of agarose gel-isolated DNA fragments derived from the *pol* genes of these viruses. These were 4.0-kb *Sst*I–*Eco*RI and 1.3-kb *Pst*I restriction fragments for HIV (Hahn *et al.*, 1984; Ratner *et al.*, 1985) and visna virus (Molineaux and Clements, 1983; Braun *et al.*, 1987), respectively. Isolation of the BIV *pol*-specific fragment is described under Results.

Heteroduplex mapping and electron microscopy

Heteroduplexes were prepared using methods previously described (Gonda, 1988). Briefly, a mixture of linear DNA (0.1 μg each) in a 10 m*M* Tris–HCl, 1 m*M* EDTA (pH 7.2) solution was denatured in 0.1 *N* NaOH for 10 min at 37°. The alkali-denatured DNA was neutralized by the addition of 0.2 vol of 1 *M* Tris–HCl (pH 7.0). Deionized formamide was added to a final concentration of 50% and renaturation at room temperature was permitted for 15–30 min. Heteroduplexes were mounted for electron microscopy by the basic protein film technique using cytochrome *c* (30–50 μg/ml) as the carrier protein in a hyperphase containing 100 m*M* TES (*N*-Tris-hydroxymethyl-2-amino ethanesulfonic acid), pH 8.5, 10 m*M* EDTA, and, to vary the stringency, 50–80% formamide. Heteroduplexes were examined and photographed in a Hitachi H-7000 electron microscope operated at 50 kV. Thin-section electron microscopy was performed as described (Gonda *et al.*, 1985).

Subcloning of BIV proviruses

λ BIV clones 106 and 127 were digested with *Sma*I, subjected to electrophoresis in agarose, transferred to nitrocellulose according to the method of Southern (1975), and hybridized with a ^{32}P-radiolabeled cDNA made to BIV RNA. Major hybridizing bands of 9.6 and 10.5 kb (BIV clones 106 and 127, respectively) were detected. Similar sized bands detected by ethidium bromide staining in a parallel experiment were excised, electroeluted, and subcloned into the *Sma*I site of the plasmid Bluescript (Stratagene) for further propagation and characterization. These clones contain most of the viral genome and, in addition, some cellular flanking sequences outside the 3′-most viral sequences (see Fig. 3).

Restriction enzyme analysis of proviral DNA

λ and plasmid BIV cloned DNAs were singly or doubly digested with various restriction enzymes under conditions suggested by the manufacturers (Bethesda Research Laboratories or New England Biolabs) and analyzed as previously described (Gonda *et al.*, 1982). In some experiments, digested DNAs were subjected to electrophoresis and transferred to nitrocellulose as described above. Virus-specific bands on filters were detected by hybridizing ^{32}P-radiolabeled cDNA prepared to BIV-specific RNA or a nick-translated probe (Rigby *et al.*, 1977) made from the subcloned 9.6-kb *Sma*I fragment of BIV 106.

Microinjection

A modified version of the microinjection technique of Diacumakos (1973) was used, as previously described (Boyd, 1985). For individual experiments, 100–200 cells were microinjected in the nucleus with DNA at a concentration of 30–50 ng/μl, and each experiment was repeated a minimum of four times.

RESULTS

Initial library screening

A library aliquot of 2.8×10^6 recombinant phage was screened for hybridization to a cDNA probe made from BIV viral RNA. Eighteen cDNA-hybridizing plaques were isolated as potential BIV proviral clones. Previous work (Gonda *et al.*, 1985, 1986) has shown that a highly conserved segment of the *pol* gene of various lentiviruses (HIV, visna virus, caprine arthritis encephalitis virus, and equine infectious anemia virus) will cross-hybridize under conditions of low stringency (washed in 0.1× SSC at 37°). DNA from the 18 potential BIV clones was purified, spotted onto nitrocellulose filters, and hybridized under these conditions with the *pol*-specific probes of HIV-1 or visna virus described under Materials and Methods. Only two λ clones, 56 and 39, hybridized with these heterologous probes. Heteroduplex mapping of these clones to each other showed approximately 6.0 kb of homologous DNA that remained annealed at high stringency (Fig. 1A). We presumed that this homologous segment represented a portion of a BIV provirus. The proviral element was truncated at one end in one clone but in the other clone was flanked on both sides by nonhybridizing segments

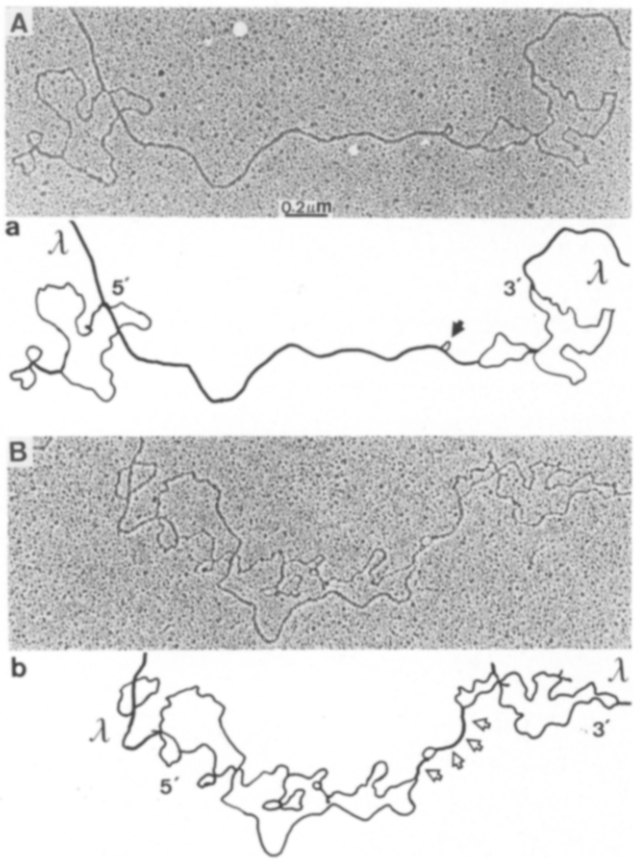

FIG. 1. (A) Heteroduplex analyses of BIV clones 39 and 56 and (B) between BIV clone 56 and visna virus (Molineaux and Clements, 1983). Heteroduplexes were prepared with inserts in the λ vector EMBL 3 (clones 39 and 56) or λ WES. (B) Visna clone. (A) and (B) are actual heteroduplexes; (a) and (b) are interpretive drawings. The stringency of hybridization, which was calculated using formulas presented in Gonda (1988), was T_m −42°. Thin and thick lines in (a) and (b) are single- and double-stranded DNA, respectively. The 5′ and 3′ ends of the inserts are indicated as well as the arms of the λ cloning vector. The solid arrow in (a) indicates the position of the deletion/insertion loop. The *pol* cross-hybridizing regions in (b) are indicated by the open arrows.

presumed to be derived from bovine cellular sequences. Additionally, the proviral element in one clone was interrupted by a 500-bp deletion/substitution loop (Fig. 1A).

Low stringency heteroduplex mapping with cloned genomes of visna virus (Fig. 1B) and HIV (data not shown) demonstrated that clones 39 and 56 shared about 1 kb of cross-hybridizing sequences with these two viruses in a similar pattern. This hybridization mapped to the amino terminus of the *pol* region of both HIV and visna virus. Moreover, these heteroduplexes demonstrated that clone 39 was truncated and that clone 56 had the potential to be a full-length provirus. Attempts to demonstrate biological function of the proviral sequences in clone 56 by microinjection or transfection of the cloned DNA into BIV-susceptible cells were unsuccessful. In later heteroduplexes formed with biologically active clones described below, we found that the 500-bp deletion seen in heteroduplexes between clones 56 and 39 definitely resided in clone 56 (data not shown).

Derivation of a BIV *pol* probe

At this point, it was clear that a *pol*-specific probe would be useful in our attempts to isolate an infectious BIV proviral clone because it would hybridize only to those clones containing the central portion of the provirus. Such clones would be a subset of all provirus-containing clones and would be more likely to be full length than clones selected by a probe representing the entire BIV genome.

We isolated a BIV *pol*-specific probe by first constructing a random shotgun library of sonication fragments from BIV clone 56 in the M13 vector mp18 (Deininger, 1983; Norrander *et al.,* 1983). We then screened this M13 library for clones hybridizing at low stringency to *pol* probes from HIV and visna virus. Twenty clones were identified, isolated, and sequenced. Most of these clones overlapped, establishing the sequence of the highly conserved core reverse transcriptase domain at the amino terminus of the BIV-*pol* gene (Gonda *et al.,* 1987). This sequence conclusively demonstrated that BIV clone 56 contains a retroviral provirus, that this provirus was not derived from any previously sequenced retrovirus, and that BIV belongs to the lentivirus subfamily. We subcloned a 420-bp *Kpn*I–*Bam*HI fragment from one of the M13 sequencing clones into the plasmid vector Bluescribe M13+ (Stratagene) for use as a BIV *pol*-specific probe. This fragment extends from an upstream *Kpn*I site across the core reverse transcriptase domain and terminates at a *Bam*HI site in the M13 polylinker.

Isolation of biologically active proviral clones

The 420-bp BIV *pol*-specific probe was used to screen an additional 1×10^7 recombinant phage from the same library of BIV-infected EBTr cell DNA. Twenty-one plaques positive for *pol* hybridization were isolated. The position and size of proviral elements in these clones were mapped by heteroduplex formation with BIV clone 56 and with each other. The heteroduplexes revealed defects in most clones, such as deletions, truncations during provirus insertion, and truncations during cloning that would prevent biological function. However, two λ clones, 106 and 127, had proviral elements that formed heteroduplexes greater than 8.0 kb in length with BIV clone 56 and approxi-

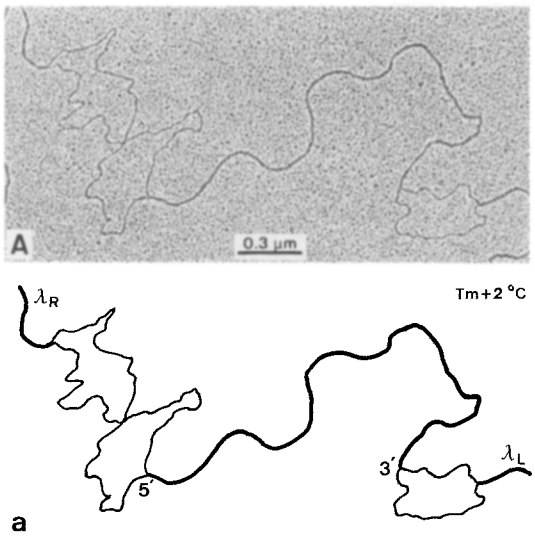

FIG. 2. Heteroduplex analysis of biologically active BIV clones 106 and 127. Heteroduplexes were prepared with inserts in the λ vector EMBL 3. (A) Actual heteroduplex; (a) interpretive drawing. The calculated stringency of the spreading solution hybridization (Gonda, 1988) was T_m +2. The 5′ and 3′ ends of the inserts are indicated as well as the arms of the λ cloning vector. In (a) the double-stranded homologous sequences in the insert are shown as thick lines and the single-stranded nonhomologous flanking sequences are represented by thin lines.

mately 9.0 kb in length with each other (Fig. 2). The proviruses in these clones were flanked on both ends by unrelated cellular sequences and therefore appeared to be good candidates for full-lenth infectious units.

Restriction enzyme site maps were inferred from single- and double-enzyme digests of DNA from BIV clones 106 and 127 (Fig. 3). In agreement with the heteroduplexing results, the two clones are approximately 9.0 kb in length and share 13 of 14 sites mapped within the provirus (a unique *Eco*RI site resides in the 3′ half of the BIV 127 genome), but differ at all sites mapped in the cellular flanking sequences. Although all sites conserved in the provirus restriction enzyme maps appear to be the same in both clones, electrophoresis experiments demonstrated that a 1.2-kb 3′ *Kpn*I segment (*Kpn*I segment from 5.3 to 6.5 kb in the BIV 127 provirus map shown in Fig. 3) was consistently 100 bp larger in the BIV 127 plasmid subclone than the corresponding segment in the BIV 106 plasmid subclone. Moreover, the size of the *Kpn*I–*Eco*RI segment (map positions 6.5 and 6.8 kb, respectively, in BIV 106 and 127) remained the same size (300 bp) in both clones. The unexpected size difference suggests that the BIV 127 provirus contains approximately 100 bp more information than BIV 106. Since no other migration anomalies were noted between the two clones in other areas of the provirus, we deduced that the additional sequences in BIV 127 reside between the *Hin*dIII and *Kpn*I sites (BIV 127 provirus map positions 5.8 and 6.5 kb, respectively; Fig. 3) and probably in close proximity to the unique *Eco*RI site located in the 3′ half of the genome.

The unique *Eco*RI site in clone 127 allowed us to go back to the original virus stock and diagnostically determine which of the two clones, if either, was the predominant provirus species relative to *Eco*RI. Total genomic DNA of cells independently infected with BIV parental stock, clone 106, or clone 127 was digested with *Eco*RI. In Southern transfers of these DNAs, using the entire 9.6-kb *Sma*I fragment of BIV 106 as the probe, we found the parental stock and clone 106 to have an identical *Eco*RI virus-specific band pattern, which was distinct from that of clone 127, suggesting that BIV clone 106 is the predominant species in the parental stock (data not shown).

Biological activity of λ BIV clones 106 and 127

Uncut purified λ DNA containing clone 106 or 127 proviral sequences was microinjected into bovine embryonic spleen (BESp) cells that had been seeded at moderate density onto glass coverslips. One hundred to two hundred cells were injected on each coverslip. At 24–48 hr after injection, as the cell sheet grew to confluence, syncytium formation could be detected between adjacent cells. These syncytia typically had 5–20 nuclei, surrounded by large, flattened cytoplasmic sheets (Fig. 4). No syncytia were seen in uninjected control cells or in cells injected with clone 56 or any of seven other defective clones (data not shown).

After syncytia formation had reached its peak (Day 4), the coverslips in dishes were overlaid with uninfected cells or were placed in T-25 flasks containing subconfluent cultures of BESp cells to amplify the infection. Supernatant fluids from these cultures were positive for reverse transcriptase activity when assayed 10 days after microinjection, while uninjected cells, or cells injected with defective proviruses, remained negative. The ability to form syncytia and supernatant reverse transcriptase activity could be passed to other primary bovine cell cultures, but not to human cells, by cell-free supernatants from microinjected cultures. Although subjectively obtained, the only biologically significant difference between the two clones was the aggressiveness with which the cell-free infection spread. BIV 106, in comparison to clone 127, tended to have more extensive and rapid syncytia formation in BESp cells, although virus recovered from cultures infected in parallel with titered stocks showed no quantitative differences in reverse transcriptase activity. Nevertheless, both BIV 106 and 127 are more

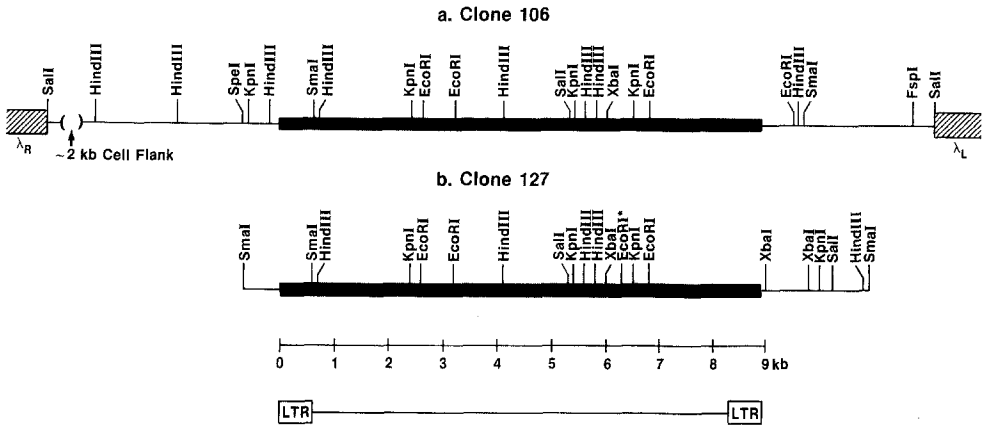

FIG. 3. Restriction enzyme site map of BIV clone 106 and 127. (a) λ BIV clone 106. (b) λ BIV clone 127. *Sal*I, *Sma*I, *Cla*I, *Hin*dIII, *Eco*RI, *Kpn*I, *Xba*I, *Spe*I, and *Fsp*I were used to map clone 106; clone 127 was mapped using all but *Spe*I and *Fsp*I. *Cla*I is a noncutter in both clones and therefore is not represented on the map. The entire insert in clone 106 was mapped in relation to the *Sal*I sites in the polylinkers at either end of the insert. Clone 127 was mapped to the first *Sma*I site in the right and left flanking sequences. The hatched areas and thin and bold lines are the λ arms, flanking DNA, and deduced BIV-specific proviral DNA sequences, respectively. The asterisk denotes the unique *Eco*RI site in clone 127 not represented in clone 106. The proviral genomes of clones 106 and 127 are each approximately 9.0 kb in length. Both clones are in the reverse orientation relative to the transcription of the cloning vector λ. This orientation was determined from the heteroduplexes between clones of BIV and visna virus or HIV whose orientations are known (Molineaux and Clements, 1983; Gonda *et al.*, 1985). The plasmid subclones of BIV 106 and 127 were derived from segments obtained from clones 106 or 127 starting at the single *Sma*I site in the 5′ proviral sequences and extending to the *Sma*I site in the 3′ flanking sequences.

aggressive *in vitro* than parental stock from which they were cloned (data not shown).

Budding and mature virus particles could be visualized by electron microscopy in cell cultures microinjected with either clone 106 (Fig. 5) or 127. These particles closely resembled those present in uncloned BIV stock cultures (Van der Maaten *et al.*, 1972; Boothe and Van der Maaten, 1974; Gonda *et al.*, 1987).

Search for endogenous BIV-related DNA sequences

We assayed for endogenous sequences related to BIV in bovine tissues. Southern transfers of total genomic DNA from BIV-infected and uninfected cells were probed with the 9.6-kb *Sma*I fragment contained in the BIV 106 subclone. This probe detected no homologous sequences in a variety of uninfected bovine cells established from fetal tissue from different animals, including spleen (Fig. 6), brain, lung, kidney, testis, choroid plexus, thymus, liver, and umbilical cord; results with the BIV *pol*-specific probe were similar. Moreover, using the BIV *pol*-specific probe, no BIV-related sequences have been found in bovine sperm DNA or DNA or tumor tissues from various cattle with lymphosarcoma (J. Casey, personal communication). In uncut genomic DNA from BIV-infected cells, a virus-specific band migrating as a 9.0-kb linear species was detected in the hybridization experiments (not shown). This pattern of hybridization was repeated if the DNA had been digested with *Cla*I, an enzyme that does not cut within the BIV clone 106 or 127 proviruses (Figs. 3 and 6). However, when DNA from BIV-infected cells was cut with *Sma*I, the hybridizing band dropped to 8.5 kb (Fig. 6), as predicted from restriction maps of BIV clones 106 and 127 (Fig. 3).

DISCUSSION

We have isolated two distinct molecular clones of functional BIV proviruses. Upon microinjection into susceptible cells, these clones mimic the biological activity of BIV virus stocks, including the induction of syncytia, reverse transcriptase activity, virus particle formation, and cell-free transmission. The two clones (106 and 127) are independent isolates in that the proviruses are flanked by different cellular sequences, as shown by heteroduplex mapping. However, the two proviruses themselves are highly related, because their heteroduplexes show no deletion/insertion or replacement loops even at high stringency, and they share 13 of 14 restriction sites mapped within the provirus. Differences between these proviruses are likely to be limited to single base substitutions and deletions or insertions less than 100 bp, about the limit of resolution of topographical features in heteroduplexes detected by electron microscopy (Gonda, 1988). These differences will be readily resolved in detailed sequence comparisons.

Lentiviruses display a remarkable amount of genetic variability (Narayan *et al.*, 1977; Payne *et al.*, 1984;

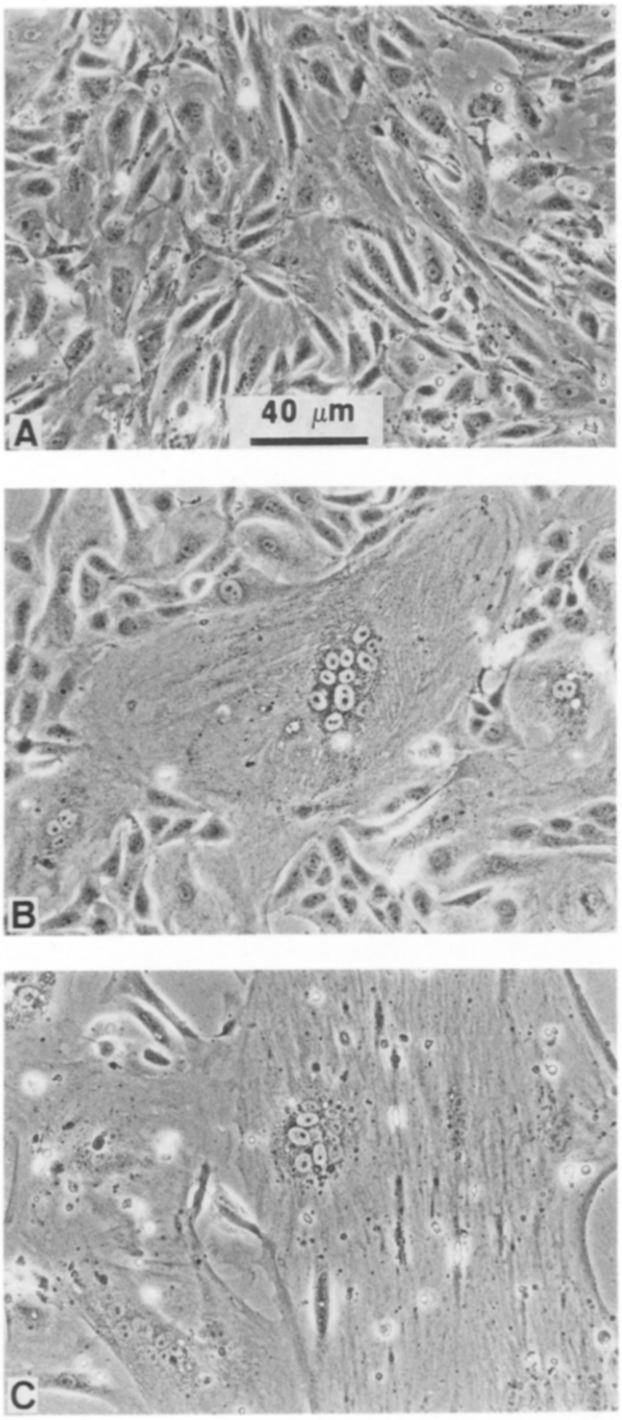

FIG. 4. Microinjection of BIV clones 106 and 127 into bovine embryonic spleen (BESp) cells. (A) Mock-injected BESp control cells. (B) Cells injected with BIV clone 106. (C) Cells injected with BIV clone 127. The photographs were taken 48 hr after microinjection of the DNA into the nucleus.

Hahn *et al.,* 1986; Braun *et al.,* 1987). In visna virus antigenic variants, there is a clustering of mutations in the envelope (Braun *et al.,* 1987). The variability is thought to play a role in the viruses' ability to escape

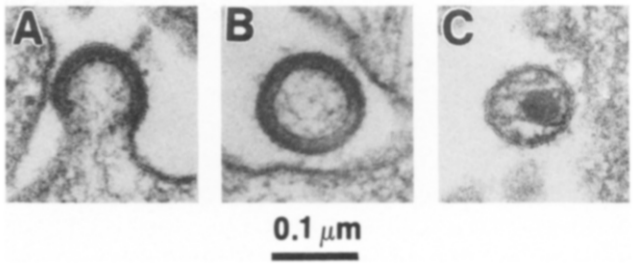

FIG. 5. Electron micrographs of BIV produced in BESp cells microinjected with clone 106 λ DNA. (A) Immature budding particle. (B) Immature extracellular particle. (C) Mature extracellular particle with typical bar- or cone-shapecd core.

the host immune response. It is interesting that the single polymorphic restriction enzyme site between clones 106 and 127 resides within the 3′ half of the genome, probably within the region coding for the viral envelope proteins. There may be additional changes, that were not detected by the limited number of restriction endonucleases used in the mapping, which may have a role in antigenic variability and pathogenesis of BIV. It will be important to assess the biologic significance of this and other differences uncovered by the sequence analysis of these two infectious clones.

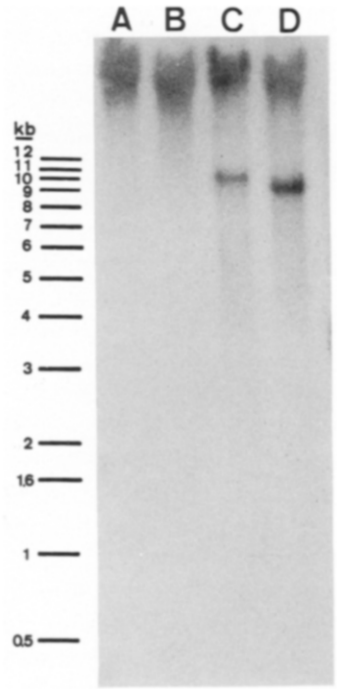

FIG. 6. Detection of endogenous BIV-related sequences by Southern transfers of total genomic bovine cell DNA. Lanes A and B are uninfected BESp cell DNA; lanes C and D are BIV-infected BESp DNA. The probe was the subcloned 9.6-kb *Sma*I fragment from BIV clone 106 (Fig. 3). Lanes A and C were cut with *Cla*I; lanes B and D were cut with *Sma*I. *Cla*I and *Sma*I are infrequent cutters in bovine DNA. *Cla*I does not cut within the BIV proviral genome and *Sma*I cuts only once, near the 5′ terminus of the proviral sequences (Fig. 3).

Southern analyses of genomic DNA from uninfected bovine cells did not detect endogenous BIV-related sequences; this indicates that BIV is an exogenous virus. In this regard, BIV resembles other lentiviruses but differs from many retroviruses of the oncovirus subfamily, for which related sequences are integrated in the germ line and are passed vertically in their hosts. The exogenous nature of BIV suggests that it is horizontally transmitted and amenable to vaccine control measures. Although exogenous, BIV is capable of provirus integration as indicated by our molecular clones in which BIV-related sequences are regularly flanked by unrelated sequences presumably derived from bovine chromosomal DNA. Undoubtedly, the ability to integrate and remain latent further enhances the viruses' ability to evade the immune response (Haase, 1986).

The importance of provirus formation in the life cycle of BIV is not known; however, a rough idea of its frequency can be derived. Assuming that bovine cells contain about 3×10^9 bp of DNA and that the average recombinant clone in our library contained 15–20 kb of bovine DNA, the 1.28×10^7 clones screened represent roughly 50 genome equivalents of bovine DNA. From this screening we isolated only 23 provirus-containing clones (although proviral elements not containing *pol* would have escaped detection), for an apparent ratio of approximately one provirus for every two genome equivalents of DNA screened. Also pertinent is the fact that the library DNA came from EBTr cells that had been mass-infected with BIV for 4 days. While such cultures show extensive cytopathic effects of the virus, many cells (50% or more) appear to remain uninvolved. These cells are probably uninfected or may not be susceptible to infection; their presence would tend to lower the apparent integrated provirus:cell ratio. Therefore, it seems likely that proviruses are integrated into the DNA of most infected cells.

While provirus integration is probably occurring in most infected cells, the proportion of defective proviruses appears high. From heteroduplex data, 6 of 23 proviral clones showed evidence of defects that were not immediately attributable to the cloning process. These defects included deletions, replacement of sequences within the provirus, and truncations of proviruses that were still flanked on both sides by bovine cellular sequences. Error-prone integration may be a property common to many retroviruses (e.g., Mullins *et al.*, 1981).

The seroprevalence of BIV in cattle populations is at present unknown and is one of the important questions about the biology of BIV that urgently needs to be addressed. A sensitive and reliable diagnostic test for BIV, like the ELISA and Western blots developed for HIV, may not be attainable using virus purified from tissue culture as was done with HIV for several reasons. First, we lack an adequate *in vitro* bovine cell system to obtain persistently infected cells that continuously produce BIV in quantities sufficient to do large-scale sampling. Second, cattle are widely infected by a variety of adventitious viral agents, one of the most common being bovine viral diarrhea virus, that often are contaminants in the calf serum used to grow bovine cells. Thus, maintaining pure stocks of virus is difficult. The biologically active molecular clones of BIV and virus stocks derived from them will be of utility in engineering the production of pure reagents through recombinant DNA technology and/or maintaining clean stocks of virus for the development of assays for seroepidemiologic studies. Furthermore, they represent well-defined tools with which to dissect the life history and pathogenesis of BIV. Their study should yield information pertinent to controlling lentiviral diseases of animals and may have relevance to HIV infection and AIDS in humans.

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