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NUCLEOTIDE SEQUENCE ANALYSIS OF THE 3′ HALF OF THE GENOME OF BOVINE LEUKAEMIA VIRUS GROWN IN FLK CELLS

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Summary. — The DNA sequence of the *env* and px (*tat*) regions of bovine leukaemia virus cloned from integrated proviral DNA of the virus producing FLK cell line was determined and compared with published sequences cloned from bovine leukaemia virus-induced tumours of cattle. The homology of 97% between a Belgian tumour clone and the FLK clone was significantly lower than that between a Japanese tumour clone and the FLK clone, where less than 1% nucleotide exchanges were observed. The sequences of cDNAs synthesized from purified virus RNA material which had been grown in different FLK sublines were found completely identical with one another as well as with the equivalent FLK proviral DNA sequences.

Key words: *bovine leukaemia virus*; *amino acid exchanges*; *env*; *tat*; *LTR*

Introduction

Bovine leukaemia virus (BLV), an exogenous retrovirus, is the causative agent of the enzootic bovine leukosis (EBL) (Burny *et al.*, 1980; Burny *et al.*, 1985). One up to several copies of proviral BLV DNA are present in tumour cells of cattle with lymphosarcoma, in the lymphocytes from infected but clinically healthy cattle or from animals with persistent lymphocytosis (Ferrer *et al.*, 1980; Kettmann *et al.*, 1982) as well as in different foetal lamb kidney (FLK) cell lines (Altaner *et al.*, 1985; Nyakatura *et al.*, 1985). The proviral DNA was found to be integrated at different sites (Kettmann *et al.*, 1980; Nyakatura *et al.*, 1985) in different mammalian cells including human myeloma cells (Sláviková *et al.*, 1986; 1987). The mechanism of the BLV-induced leukaemogenesis is unknown.

Although the DNA sequences of complete proviruses cloned from both a Japanese and a Belgian bovine tumour case were published (Rice *et al.*, 1984; Rice *et al.*, 1985; Sagata *et al.*, 1985*a*) there is little information con-

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cerning the variability of the BLV genome. For comparison, therefore, we have determined the nucleotide sequence of BLV clone (λ-BLV HU1, Nyakatura *et al.*, 1985) originating from permanently infected FLK cells. This cell line was first established by Van Der Maaten and Miller (1976) and since than it has been widely used in BLV research. Up to now only some of the 3′ terminal sequences have been published (Tsimanis *et al.*, 1983; Derse *et al.*, 1985; Ivanova *et al.*, 1986; Rosen *et al.*, 1986; Brantl *et al.*, 1988). Here we describe nucleotide and amino acid exchanges between the sequences of λ-BLV HU1 and the Belgian and Japanese tumour clones, resp., of the 3′ half of the genome starting with the *env* region. In addition, we have studied the structural features of the deduced gene products, especially of the major surface antigen gp51.

Materials and Methods

The cloning of λ-BLV HU1 has been reported (Nyakatura *et al.*, 1985). This clone terminates within host-flanking sequences on its 5′ end and within the *Eco*RI site 200 bp upstream from the end of the 3′ LTR. It was found to harbour 8.1 kb viral sequences flanked by a 2.4 kb cellular stretch at the 5′ end. The fragments of λ-BLV HU1 produced by digestion with *Bam*HI, *Hind*III, *Sma*I, and *Eco*RI, resp., were cloned into the plasmids pUC13 or pUC19 to obtain pEB19-1/2 (2 400 nucleotides of the 5′ terminal cellular sequence through position 2 038), pBB13-3 (from position 2 039 through 5 225), pBB13-4 (from position 5 226 through 7 202), pHM19-3/4 (from position 4 116 through 5 712), and pBE19-5 (from position 7 203 through 7 924). For comparison with other published sequences we used the numbering according to Sagata *et al.* (1985*a*). The sequences representing the 3′ half of the BLV genome were cut out with *Xho*I, *Sma*I, *Bgl*II, *Pvu*II, *Eco*RI, and *Bam*HI. The 5′ terminal region was digested with *Sst*I, *Bam*HI, and *Eco*RI to clone the 5′ LTR. The resulting fragments were subclone into M13 mp18/mp19.

The cloning of cDNA from BLV produced in FLK cell culture has been reported (Rosenthal *et al.*, 1984). The 3′ cDNA clones pLK166, pLK232, and pLK520 were obtained by cDNA synthesis using oligo dT as the primer. The double-stranded cDNA fragments were cloned into pBR322 at the *Pst*I site after dC and dG tailing of the target and vector DNAs, resp. The inserts were subcloned into M13 mp18/mp19. Furthermore, the insert of the cDNA clone pLV10 constructed in the same manner by Tsimanis *et al.* (1983) was also subcloned into M13 mp18/mp19.

Selected clones were sequenced by the chain termination method according to Sanger *et al.* (1977). The nucleotide sequences read from the autoradiogrammes were computed using programmes constructed by J. Winkler and by one of us (B.D.). The amino acid sequences encoded by two large reading frames, *env* and *tat*, were predicted and the substitutions were counted by comparison with the known proviral sequences of the Japanese and the Belgian isolates.

Results and Discussion

When taking the sequence published by Sagata *et al.* (1985*a*) for basis, the *env* gene of λ-BLV HU1 spans nucleotides 4 821—6 368 and presumably encodes 515 amino acids (signal peptide, gp51 and gp30). The gene coding for gp51 consists of 804 nucleotides. Six nucleotides (five in the third position of the respective codons) differ between the Japanese tumour clone and the λ-BLV HU1 clone (0.7%), and nucleotides (20 in the third position of the codons) differ between the Belgian tumour clone and the λ-BLV HU1 clone (3.7%) resulting in one and eight amino acid exchanges, respectively (Table 1). One and five, respectively, of the exchanged residues are favoured

Table 1. Nucleotide and amino acid exchanges of gp51 of BLV

Amino acid residue	Belgian clone		λ-BLV HU1		Japanese clone	
8	AA**T**	(Asn)	AA**C**	(Asn)		
15	A**C**A	(**Thr**)	**G**CA	(**Ala**)		
30	**T**TA	(Leu)	**C**TA	(Leu)		
33	CA**C**	(His)	CA**T**	(His)		
36	CA**C**	(His)	CA**T**	(His)		
35	CA**A**	(Gln)	CA**G**	(Gln)		
40	**C**CC	(**Pro**)	**G**CG	(**Ala**)	GC**C**	(Ala)
41	AG**G**	(**Arg**)	AA**G**	(**Lys**)		
49	**T**TC	(**Phe**)	**T**C**T**	(**Ser**)		
52	GG**T**	(Gly)	GG**C**	(Gly)		
57	TA**T**	(Tyr)	TA**C**	(Tyr)		
88	CA**C**	(His)	**C**G**C**	(**Arg**)	CG**G**	(Arg)
100			G**C**C	(Ala)	GC**T**	(Ala)
103	GG**G**	(Gly)	GG**A**	(Gly)		
116	CT**C**	(Leu)	CT**T**	(Leu)	CT**C**	(Leu)
128	GA**A**	(Glu)	GA**G**	(Glu)		
142	AA**A**	(Lys)	AA**G**	(Lys)		
143	AT**T**	(Ile)	AT**C**	(Ile)		
151	TT**C**	(Phe)	TT**T**	(Phe)		
152	CC**T**	(Pro)	CC**C**	(Pro)		
154	**C**TG	(Leu)	**T**TG	(Leu)		
170	AA**T**	(Asn)	AA**C**	(Asn)	AA**T**	(Asn)
172	AC**G**	(Thr)	AC**A**	(Thr)		
202	**G**GC	(**Gly**)	**A**GC	(**Ser**)		
206	GG**T**	(Gly)	GG**C**	(Gly)		
219			AC**G**	(**Thr**)	**T**CG	(**Ser**)
221	TTG	(**Leu**)	TC**G**	(**Ser**)		
245	TT**A**	(Leu)	TT**G**	(Leu)		
248	CC**C**	(Pro)	CC**T**	(Pro)		
258	GT**T**	(**Val**)	G**C**T	(**Ala**)		
268	CG**C**	(Arg)	CG**T**	(Arg)		

λ-BLV HU1 was compared with the Belgian and Japanese tumour clones. Differences between the Belgian and Japanese tumour clones are not indicated. Exchanged neucleotides and amino acids are in bold type. The numbering of the amino acid residues starts from the N-terminus of the mature protein.

(conservative) substitutions. If defined as pairs of residues, both belong to the same amino acid group (Schwartz and Dayhoff, 1978). The point mutations resulting in amino acid exchanges are distributed as clusters within two relatively short domains. The first domain (amino acid positions 15—88) contains five such substitutions and the second one (positions 202—258) four substitutions (Fig. 1).

The gene coding for the transmembrane protein gp30 consists of 642 nucleotides (Table 2). Two nucleotides in the third position of the codons were found to be replaced in the Japanese tumour clone (0.3%) without any amino acid substitution, whereas 18 nucleotides (14 at the third position

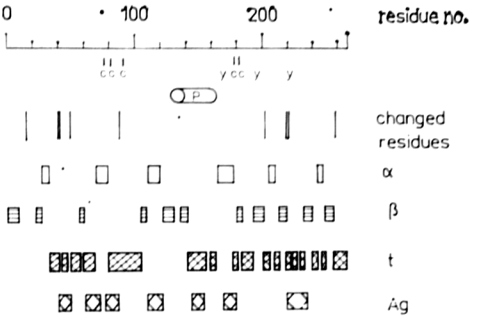

Fig. 1.
Secondary structure analysis of gp51 of BLV
C = cystein; Y = potential site of glycosylation; P = proline-rich region; α = α-helix; β = β-sheet; t = β-turn; Ag = antigenic determinant.

of the respective codons) were replaced in the Belgian tumour clone (2.8%) resulting in three amino acid exchanges near to the C-terminus (one of which is a favoured substitution).

The px (*tat*) region contains an open reading frame consisting of 924 nucleotides which encode a presumed transforming protein (Sagata *et al.*, 1985*b*). Since the *Eco*RI site used for cloning is located 200 bp upstream from the 3′ LTR within the px gene, we were not able to sequence completely this gene from the proviral clone λ-BLV HU1. Nevertheless, the sequence comparison of the λ-BLV HU1 clone with the Japanese and the

Table 2. Nucleotide and amino acid exchanges of gp30 of BLV

Amino acid residue	Belgian clone		λ-BLV HU1		Japanese clone	
4			GC**A**	(Ala)	GC**G**	(Ala)
18			GG**A**	(Gly)	GG**C**	(Gly)
21	GT**A**	(Val)	GT**G**	(Val)		
50	AA**C**	(Asn)	AA**T**	(Asn)		
53	CA**T**	(His)	CA**C**	(His)		
63	GT**C**	(Val)	GT**T**	(Val)		
86	AT**C**	(Ile)	AT**T**	(Ile)		
88	GA**A**	(Glu)	GA**G**	(Glu)		
93	CT**A**	(Leu)	CT**G**	(Leu)		
128	AC**C**	(Thr)	AC**T**	(Thr)		
141	CT**A**	(Leu)	CT**G**	(Leu)		
151	**T**TG	(Leu)	**C**TG	(Leu)		
165	AA**A**	(Lys)	AA**G**	(Lys)		
178	TT**C**	**(Phe)**	TT**A**	**(Leu)**		
179	**C**CC	**(Pro)**	AC**C**	**(Thr)**		
191	CC**G**	(Pro)	CC**A**	(Pro)		
192	TC**A**	(Ser)	TC**T**	(Ser)		
203	AC**C**	**(Thr)**	**G**TC	**(Val)**		
210	CT**T**	(Leu)	CT**C**	(Leu)		

For explanation see legend to Table 1.

Table 3. Nucleotide and amino acid exchanges of px(BL)I

Amino acid residue	Belgian clone		λ-BLV HU1		Japanese clone	
36	GA**A**	(Glu)	GA**G**	(Glu)		
55	A**C**C	(**Thr**)	A**A**C	(**Asn**)		
63	**TTT**	(Pha)	**TGC**	(**Cys**)		
68	A**C**A	(Thr)	A**C**G	(Thr)		
109	**C**AG	(**Gln**)	**C**GG	(**Arg**)		
129	AT**C**	(Ile)	AT**T**	(Ile)		
147	**G**GA	(**Gly**)	A**G**A	(**Arg**)	**G**GA	(**Gly**)
163	**C**CC	(Pro)	**T**CC	(**Ser**)		
169	GT**A**	(Val)	GT**G**	(Val)		
179	CC**G**	(Pro)	CC**A**	(Pro)		
181	A**C**T	(Thr)	A**C**C	(Thr)		
196	C**C**A	(Pro)	C**C**G	(Pro)	C**C**A	(Pro)
197	G**T**T	(**Val**)	G**C**T	(**Ala**)		
218	CG**A**	(Arg)	CG**G**	(Arg)		
220	A**C**C	(**Thr**)	**T**CC	(**Ser**)		
221	A**G**T	(**Ser**)	AAT	(**Asn**)		
223	CC**T**	(Pro)	CC**C**	(Pro)		
232			C**T**T	(**Leu**)	C**C**C	(**Pro**)
			cDNAs			
256	TA**C**	(**Tyr**)	T**G**C	(**Cys**)	T**G**T	(**Cys**)
261	GT**G**	(Val)	GT**A**	(Val)		
269	CA**A**	(Gln)	CA**G**	(Gln)		
272	CT**C**	(Leu)	CT**T**	(Leu)		
277	CT**C**	(Leu)	CT**A**	(Leu)		
280	TC**A**	(Ser)	T**C**C	(Ser)		
282	TT**G**	(Leu)	TT**A**	(Leu)	TT**G**	(Leu)
286	**GGG**	(**Gly**)	**A**GA	(**Arg**)		
288	C**T**A	(**Leu**)	**A**TA	(**Ile**)		
292	TC**A**	(Ser)	T**C**C	(Ser)		
297	CA**G**	(Gln)	CA**A**	(Gln)		
299	**T**TA	(Leu)	**C**TA	(Leu)		

For explanation see legend to Table 1. The numbering starts from the N-terminal residue encoded by the large open reading frame within *tat*.
px(BL)I and px(BL)II designate different reading frames.

Belgian tumour clones showed that two out of 684 (0.3%, one at the third position) and 18 out of 684 (2.6%, 10 at the third position) nucleotides were replaced, resulting in one (0.4%) and eight (3.5%) substitutions of amino acids, respectively. Of the latter two are favoured ones (upper part of Table 3). With the exception of the N-terminus, where the open reading frame overlap, the amino acid substitutions seem to be randomly distributed. The lower part of Table 3 gives the nucleotide and amino acid substitutions as seen with the sequence completing cDNAs synthesized with viral RNA templates from FLK cell cultures. Rosen *et al.* (1986) published the sequence of a cDNA clone representing the *tat* gene of BLV from an FLK

Table 4. Nucleotide and amino acid exchanges of px(BL)II

Amino acid residue	Belgian clone		λ-BLV HU1		Japanese clone
53	AAC	(Asn)	AGC	(Ser)	
72	CCT	(Pro)	ACT	(Thr)	
80	TTG	(Leu)	GCG	(Ala)	
85	CAT	(His)	CGT	(Arg)	
126	AAT	(Ser)	GGT	(Gly)	
146	TCA	(Ser)	TTA	(Leu)	

For explanation see legend to Table 1. The numbering starts from the N-terminal residue encoded by the short open reading frame within *tat*.
px(BL)I and px(BL)II designate different reading frames.

cell line. Interestingly, when compared with the other sequences, this sequence was identical to the corresponding sequence of the Japanese tumour clone. Therefore, we believe that the FLK cell line used by these authors should be more related to the Japanese tumour clone than our FLK cell line.

In addition to the major open reading frame the *tat* gene of BLV contains a short open reading frame which overlaps this major one and encodes a protein of 156 amino acids (Sagata *et al.*, 1985*b*). The overlapping region consists of 417 nucleotides. We did not find any difference between the λ-BLV HU1 clone and the Japanese tumour clone (Table 4). The comparison with the Belgian tumour clone revealed seven nucleotide replacements (1.7%) resulting in six amino acid substitutions (4.3%, three of which are favoured ones).

Between the 3′ end of the *env* gene and the open reading frame of px (*tat*) there are 877 nucleotides which probably constitute a noncoding region (NCR). As compared with the same region of the Japanese and Belgian tumour clones, we found in it seven (0.8%) and 30 (3.4%) substituted nucleotides, respectively.

The LTR sequence of BLV consists of 630 nucleotides. For comparison we sequenced the 5′ LTR and found three different nucleotides (0.5%) in the Japanese tumour clone and 10 different nucleotides (1.6%) in the Belgian one. As compared with the published sequence of the 5′ LTR (Derse *et al.*, 1985) the λ-BLV HU1 LTR has a deletion of one nucleotide within the 5′ half of the U3 region. From these data it is obvious that in the 3′ half of U3, starting about 130 nucleotides upstream from the cap site, no differences do exist between all known sequences. In this region are located the transcriptional regulatory elements such as CAT box, TATA box, the poly(A) signal, the cap site, and a part of the enhancer (Sagata *et al.*, 1984). The cDNA clones pLK232 and pTK520 which, in respect to the nucleotide sequence, are identical with one another represent the region between the *Eco*RI site located at position 7 924 and the R region of the 3′ LTR. It is noteworthy that the 3′ termini of both cDNA clones do not represent the

poly(A) site. In both cases the 3′ terminus is found 80 nucleotides upstream from the poly(A) site exactly in the loop of the main stem of the proposed hairpin structure (Sagata *et al.*, 1984) indicating that the cDNA synthesis was influenced by this secondary structure.

Taken together, the nucleotide sequence analysis of the 3′ half of the PLV genome cloned from integrated proviral DNA of the virus producing FLK cell line revealed striking conservation when compared with the corresponding region of two BLV clones from tumour tissue. The homology to the Belgian tumour clone (97%) is significantly lower than that to the Japanese one (>99%). The first analysis of a possible genetic drift between various BLV isolates from U.S.A. and Belgium comparing p12 sequences showed that the drift could be estimated low (Burny *et al.*, 1984).

All the differences between the clones which we have investigated were single nucleotide and amino acid exchanges. Our data clearly indicate that the exchanged amino acids are not randomly distributed along the genome. We found the highest variability within two domains of gp51 (about 7% amino acid substitutions). The lowest variability we found within the LTR (0.5% and 1.6% nucleotide exchanges). It is known that the BLV proviral sequences in tumour tissues do not give rise to virus production. On the other hand we cannot decide whether or not the proviral sequence we have cloned from FLK cells produces active virus, as the cells contain at least three proviruses (Nyakatura *et al.*, 1985). The fact that our cDNAs made from FLK RNA material do not show any nucleotide exchanges when compared to our proviral clone might indicate that the respective proviral sequence belongs to an expressed provirus.

In order to characterize the mutations within gp51 we used a secondary structure prediction algorithm consisting of a modified combination of algorithms according to Novotny and Auffrey (1984) using the hydropathic analysis of Kyte and Doolittle (1982), the empirically based secondary structure analysis of Chou and Fasman (1978) and the hydrophobic moment plot of Eisenberg *et al.* (1984). We conclude that all exchanged amino acid residues are located in regions which preferentially form β-turns or loops. This is in accordance with the rule that residues on the surface vary faster than the internal ones (Schultz and Schirmer, 1979). For gp51 by means of our prediction method we estimated 25% β-pleated sheets and 17% α-helix (Fig. 1).

The knowledge of both the secondary structure and the positions and chemical nature of exchanged amino acids may be helpful to predict antigenic epitopes and to direct the choice of synthetic peptides to be tested as antigens. The search for antigenic epitopes of gp51 using the empirically based method of Welling *et al.* (1985) and the hydropathic analysis of Hopp and Woods (1981) in combination with the secondary structure prediction shows that seven sequential antigenic determinants may be found in the N-terminal region containing exchanged amino acid residues (residues 39—47, 60—70, 75—85), in the middle conserved part (residues 109—120, 144—152, 168—178), and in the C-terminal region containing exchanged residues (residues 220—235) (Fig. 1). Meanwhile we were able to localize experimen-

tally some antigenic determinants of gp51. A subfragment covering the amino acid pos. 55—102, which was expressed in *E. coli* as a fusion protein with β-galactosidase, actively reacts with sera of BLV-infected cattle in Western blot (antigenic determinants 2 and 3 from left to right — Fig. 1) (Siakkou *et al.*, 1990; Portetelle *et al.*, 1989). A synthetic peptide representing the amino acid sequence in positions 78—92 (antigenic determinant 3 — Fig. 1) reacts with some BLV-positive sera (Portetelle, personal communication), another peptide consisting of amino acids from positions 57—67 reacts with a monoclonal antibody not competing with the sera from BLV-infected cattle. Finally, monoclonal antibodies (Bruck *et al.*, 1982; Portetelle, personal communication; Platzer *et al.*, in preparation) are mainly directed against the domain represented by the amino acid sequence in positions 167—217 (antigenic determinant 6 — Fig. 1). Proteins like gp51, which are modified posttranslationally by the addition of sugar moieties, may not be achieved in a native-like conformation if synthesized in *E. coli* as a non-glycosylated polypeptide chain. The biological significance of the conserved region (residues 90—200) which includes proline rich sequences is not yet clear. However, as discussed by Koch *et al.* (1983), the proline rich regions could carry the domains utilized for the interaction with cell surface receptors.

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