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Expression of the Bovine Leukemia Virus X Region in Virus-Infected Cells

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Bovine leukemia virus, like its closest relatives the human T-cell leukemia virus types I and II, contains a 1.8-kilobase X region between the *env* gene and the 3′ long terminal repeat. In this communication, we report the detection and characterization of a subgenomic mRNA from which this X region is presumably translated. This mRNA was produced by a complex splicing mechanism which resulted in juxtaposition of the 5′ end of the *env* gene and the two overlapping X-region open reading frames. Translation of this mRNA could yield at least two distinct proteins depending on which initiation codon is used. Detection of the protein encoded by the BLV X-region long open reading frame has been reported (N. Sagata, J. Tsuzuku-Kawamura, M. Nagayoshi-Aida, F. Shimizu, K.-I. Imagawa, and Y. Ikawa. Proc. Natl. Acad. Sci. USA 82:7879–7883, 1985). Using synthetic peptide antisera, we detected a protein encoded by the short open reading frame in virus-infected cells. The protein migrated in sodium dodecyl sulfate-polyacrylamide gels with an apparent molecular weight of 19,000. It is a nuclear phosphoprotein.

The retrovirus bovine leukemia virus (BLV) is the causative agent of enzootic bovine leukosis, a disease characterized by clonal tumors of the B-cell lineage in cattle and the T-cell lineage in sheep (8, 24). The mechanism of disease induction is unknown. The virus apparently need not be expressed in tumors (23, 25) and contains no sequences able to hybridize with normal cellular DNA (15). The BLV genome is integrated in tumor DNA, but specificity in integration has not been detected (15, 22). Thus, neither an oncogene nor a promoter insertion model of tumorigenesis is particularly satisfactory.

In our recent molecular analysis of the BLV genome, we detected some unusual aspects of the sequence organization of BLV that might help us to understand the mechanism of leukemogenesis. We have determined and reported the complete nucleotide sequence of a molecular BLV clone from a bovine tumor (38–40). The sequence revealed that in addition to the *gag*, *pol*, and *env* genes found in all replication-competent retroviruses, the BLV genome contains a 1.8-kilobase (kb) region between the *env* gene and the 3′ long terminal repeat. It is unlikely that the 5′ half of this so-called X region encodes a protein, for there are multiple translation terminators in all three reading frames (39). However, there are two overlapping open reading frames in the 3′ half of X, i.e., a short open reading frame (*sor*), and a long open reading frame (*lor*). Human T-cell leukemia virus types I and II (HTLV-I and -II), which are the closest relatives of BLV (11, 12, 36, 38, 39, 45, 46), also possess an X region, and the sizes and relative positions of their *sor* and *lor* are very similar to those of BLV. HTLV-I proteins derived from both *sor* and *lor* have been reported (26, 29, 51). In previous work with BLV, X-region mRNA has been detected in virus-infected cells (30, 44) and a 38,000-molecular-weight protein which is the product of the *lor* gene has been reported (42). Like the HTLV-I and -II *lor* proteins, the BLV X-*lor* protein has *trans*-activating activity, acting either directly or indirectly to stimulate transcription from the viral long terminal repeat (9, 17, 42, 52). In the present work, we show that the *sor* gene also encodes a protein found in BLV-infected cells. It is a nuclear phosphoprotein with an apparent molecular weight of about 19,000, and it is translated from a doubly spliced mRNA.

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MATERIALS AND METHODS

Cells. The BLV-producing fetal lamb kidney (FLK) cell line was established and described by Vander Maaten and Miller (56). The BLV-producing bat lung cell line Tbl-Lu/BLV was established and described by Graves and Ferrer (21). These cells, as well as the uninfected bat line TblLu, were grown in Dulbecco modified Eagle medium plus 10% fetal calf serum at 37°C.

Preparation of RNA and Northern blots. RNA was isolated by the guanidine hydrochloride method (14), and molecules containing 3′ poly(A) tails were selected by oligo(dT)-cellulose chromatography (2) after heating at 65°C for 5 min in distilled water. Electrophoresis in methylmercuric hydroxide was carried out as previously described (3, 6, 20) by using about 5 μg of poly(A)$^+$ RNA per lane. One lane of the gel contained unfractionated RNA and was stained in ethidium bromide after electrophoresis to visualize 28S and 18S rRNAs. The remaining lanes were blotted onto nitrocellulose (55). In one experiment (see Fig. 2, lanes 4 to 7), RNA was fractionated in a formaldehyde gel (19, 31).

DNA probes. Subclones in pUC8 of a BLV tumor-derived genome (15) were digested with the appropriate restriction enzyme(s), and DNA was fractionated by electrophoresis in low-melting-point agarose gels. DNA fragments were purified by NACS-52 chromatography (Bethesda Research Laboratories, Inc.) and nick translated (41) by using [α-^{32}P]dCTP (400 Ci/mmol). Specific activity of the DNA was usually about 2×10^8 cpm/μg.

Oligomers were synthesized by using the Applied Biosystems DNA synthesizer and were purified by electrophoresis through 20% acrylamide containing 7 M urea. Oligomers were labeled at their 5′ termini by using [γ-^{32}P]ATP (3,000 Ci/mmol) and T4 polynucleotide kinase (Be-

thesda Research Laboratories). The resulting specific activity was about 1.5×10^9 cpm/μg.

Hybridization of Northern blots. Oligonucleotides were hybridized in the buffer described by Thomas (55), except that it contained no formamide or dextran sulfate. Labeled oligomer was present at 2×10^6 cpm/ml. After incubation for 24 h at 35 to 42°C, the filter strips were washed in 6× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) at 37 to 55°C, depending on the G+C content of the oligomer. Filters were exposed to X-ray film in the presence of an intensifying screen for 2 to 4 days.

Higher-molecular-weight DNA fragments were boiled and hybridized with the Northern blots for about 40 h at 42°C. The buffer (55) contained 40% formamide, no dextran sulfate, and [^{32}P]DNA at 2×10^6 cpm/ml. The most stringent wash was 0.5× SSC at 55°C. Bands could typically be seen after overnight exposure of the filter to X-ray film with no intensifying screen.

Metabolic labeling. Cells were rinsed in phosphate-buffered saline and incubated in methionine-free minimum essential medium plus 10% dialyzed calf serum for 10 min at 37°C. [^{35}S]methionine (1,000 Ci/mmol; Amersham Corp.) was added to a final concentration ranging from 25 to 100 μCi/ml, and incubation continued for 2 to 5 h. In one experiment (see Fig. 4A), cells were labeled with both [^{35}S]methionine and [^{35}S]cysteine (1,000 Ci/mmol; Amersham). These were added to medium lacking both methionine and cysteine.

To label with $^{32}P_i$, cells were rinsed in phosphate-free RPMI and preincubated for 4 h in phosphate-free RPMI containing 10% dialyzed calf serum and 50 μM sodium orthovanadate. A 1/100 volume of PBS was then added along with $^{32}P_i$ (carrier free; Amersham) to a final concentration of 200 μCi/ml. Incubation continued for about 4 h prior to processing for immunologic analysis.

Immune precipitations. Our procedure has been described in detail previously (37). Briefly, cells were lysed in buffer containing Tris, sodium chloride, EDTA, sodium deoxycholate, Triton X-100, and sodium dodecyl sulfate (SDS); and lysates were incubated with antiserum plus protein A-Sepharose (Pharmacia, Inc.). Immune pellets were boiled and loaded onto 10 to 20% gradient SDS-polyacrylamide gels (28). ^{35}S-containing gels were impregnated with 2,5-diphenyloxazole before being dried.

Synthesis of peptides and immunizations. Peptides were synthesized by conventional solid-phase techniques (4) by using a Vega Model 250C coupler. The following peptides were synthesized: peptide I (CMSPRPAPKGPDD), corresponding to residues 90 to 102 of the predicted amino acid sequence, and peptide II (PLSGTAFFPGTT), corresponding to C-terminal residues 145 to 156. Each synthesis used the standard combination of *tert*-butyloxycarbonyl derivatives for the α-amino function and benzyl-type derivatives for the side chains of trifunctional amino acids. For the addition of arginine, the tosyl derivative was used. For peptide I, which contains methionine, 1,2-ethanedithiol (0.2%) was added to the 40% trifluoroacetic acid (TFA) in dichloromethane immediately after the addition of methionine and was used in the subsequent deprotection steps. The peptidyl resins were treated with liquid HF containing 10% anisole, 3.2% dimethylsulfide, and 1.6% 1,2-ethanedithiol for 50 to 60 min to remove the protecting groups and effect the cleavage of the peptide from the resin. Crude peptides were purified by gel filtration by using G-25 Sephadex in 5% acetic acid and by preparative reverse-phase high-performance liquid chromatography by using a μBondapack C18 column (Waters Associates, Inc.). The peptide, dissolved in 7 M guanidine hydrochloride containing 0.1% TFA, was applied to the column, which was equilibrated with 0.1% TFA–10% acetonitrile in water at a flow rate of 4.0 ml/min, and was eluted with a 30-min gradient of 10 to 60% acetonitrile which also contained 0.1% TFA.

Peptides (5 mg) were coupled to bovine serum albumin (20 mg) by the method of Tager (54). This procedure results in the coupling of α-amino and ε-amino lysyl groups to the carrier protein by means of a bifunctional reagent. New Zealand White rabbits were immunized with 200 μg of peptide carrier protein conjugates intradermally by the following schedule: immunization 1, 200 μg of conjugate in complete Freund adjuvant; and immunizations 2 and 3 at 2-week intervals, 200 μg of conjugate in incomplete Freund adjuvant. At 1 week after the final immunization, rabbits were bled at weekly intervals and sera were tested by enzyme-linked immunosorbent assay against the unconjugated peptide.

Other antisera. Antisera to purified BLV gp60 (45) and p24 were gifts of Alan Schultz and Stephen Oroszlan.

Phosphoamino acid analysis. Gel slices were crushed and soaked overnight at 4°C in water containing 1 mM phenylmethylsulfonyl fluoride. The eluate was dialyzed against water, and the sample was lyophilized. After being suspended in 200 μl of 4 N HCl, the sample was heated to 110°C for 1.5 h. It was then lyophilized again after the addition of 10 nmol each of phosphoserine, phosphothreonine, and phosphotyrosine (Sigma Chemical Co.). The sample was taken up in 10 μl of electrophoresis buffer (90 ml of water, 8 ml of acetic acid, 2 ml of formic acid [pH 2.0]) and spotted on a thin-layer chromatography plate (EM Reagents; 0.1 mm). Electrophoresis was carried out at 500 V for 4 h, followed by chromatography in the second direction in 30 ml of 0.5 M ammonium hydroxide plus 50 ml of isobutyric acid (pH 3.5). The dried plate was sprayed with a solution of 40 mg of ninhydrin dissolved in 10 ml of acetone and was baked for 5 min at 60°C. Autoradiography was carried out at −70°C with an intensifying screen.

RESULTS

Detection of X-region mRNA in BLV-infected cells. To look for expression of the BLV X region, RNA was prepared from the virus-producing bat lung cell line TblLu/BLV (21). This RNA was enriched for mRNA by passage over oligo(dT)-cellulose, and aliquots of the resulting poly(A)-containing fraction were electrophoresed under denaturing conditions and blotted onto nitrocellulose. Such blots were hybridized with 1 of 10 different DNA probes, each representing a specific region of the BLV genome. The results are shown in Fig. 1.

As expected, all 10 DNA fragments hybridized with an RNA of about 8.6 kb, the complete viral genomic mRNA. An RNA of about 4.3 kb was detected by probes drawn from the 3′ half of the genome (fragments 4 to 10) but not by *gag* or *pol* probes (fragments 1 to 3). The *env* gene is presumably translated from this subgenomic mRNA. Although X-region sequences also appear in this 4.3-kb RNA, they presumably cannot be translated because of the multiple termination codons in all three frames in the 5′ half of X. A minor band at 6.2 kb was also detected with *gag*, *pol*, and X probes. It is not known whether this RNA is a spliced product transcribed from an intact BLV genome or whether it is a transcript of a genome bearing a sizable deletion.

X sequences also appeared in an RNA of about 1.9 kb. In this RNA, the region containing the multiple termination

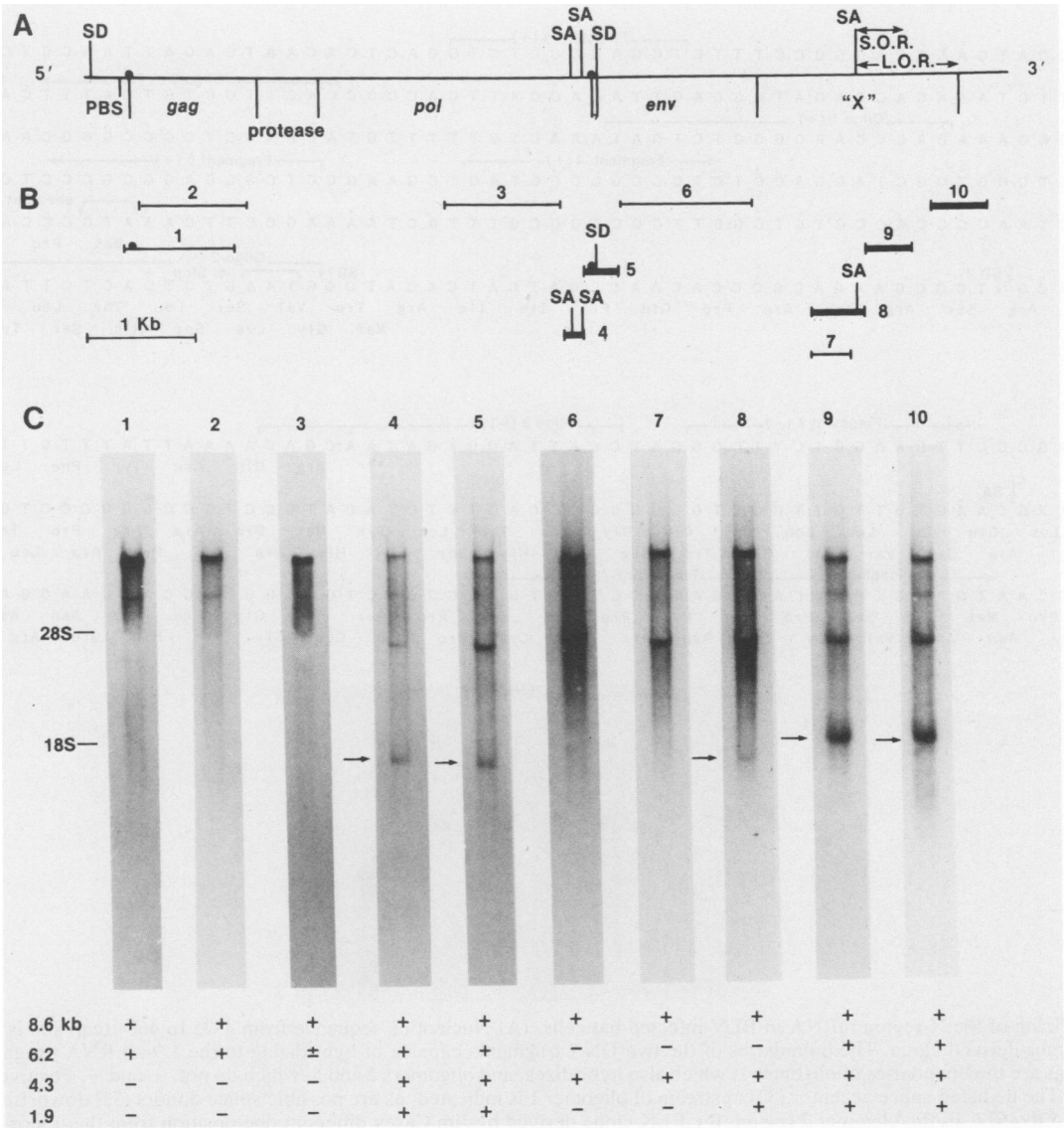

FIG. 1. Identification and characterization of BLV X-region mRNA. (A) A diagram of the 8.4-kb BLV genome based on its DNA sequence (38–40). Abbreviations: PBS, primer binding site; SD, potential splice donor; SA, potential splice acceptor; S.O.R., short open reading frame; L.O.R., long open reading frame. The *gag* and *env* initiator methionine codons are indicated by closed circles. (B) BLV DNA fragments used in RNA hybridization experiments. Those fragments which hybridized with the putative X mRNA (i.e., 4, 5, 8, 9, and 10) are drawn with heavy lines. (C) Northern blots of RNA from BLV-infected bat cells by using the probes shown in panel B. The putative X mRNA (1.9 kb) is indicated by an arrow. Sizes of hybridizing RNAs are given below each blot. RNAs hybridized with probes 6, 9, and 10 were electrophoresed for a shorter time than usual, resulting in less separation of the hybridizing bands. They have been arbitrarily aligned with the others at the position of the *env* mRNA. The RNA preparation used for hybridization to probes 1 and 3 had considerable residual rRNA, even after oligo(dT)-cellulose chromatography. The 28S RNA appears as a negative image on the Northern blots.

codons was not present (fragment 7 did not hybridize) but the region containing the open reading frames was present (fragments 8 and 9 hybridized). In addition, fragments 4 and 5 also hybridized with this 1.9-kb RNA. Fragment 4 contains several possible splice acceptors, while fragment 5 contains the initiator for the *env* gene (39). Fragment 6, representing most of the *env* gene, did not hybridize to this RNA; nor did fragment 1, which represents most of *gag* and carries the *gag* intitiator, or fragment 3, which represents most of the 3′ half of the *pol* gene. It therefore appears that as in HTLV-I and HTLV-II (1, 48, 57), two splicing events must occur to generate this 1.9-kb RNA; i.e., the 5′ cap site must be spliced to an acceptor upstream of *env*, as in the *env* mRNA, and a donor located within fragment 5 must then be spliced to an acceptor within fragment 8. This doubly spliced RNA is presumably the message from which the extreme 3′ portion of the BLV genome is translated.

Localization of splice sites in the X-region mRNA. To define the 1.9-kb RNA more precisely, we synthesized DNA oligonucleotides complementary to specific regions of viral RNA and examined their abilities to hybridize to the three major RNA size classes. Oligomers 1 and 5 are derived from fragment 4, which contains several possible splice acceptor sites (Fig. 2A). Oligomer 1 was able to hybridize with the 1.9-kb RNA, as well as with the 4.3- and 8.6-kb RNA, but oligomer 5 hybridized only with genomic RNA (Fig. 2C).

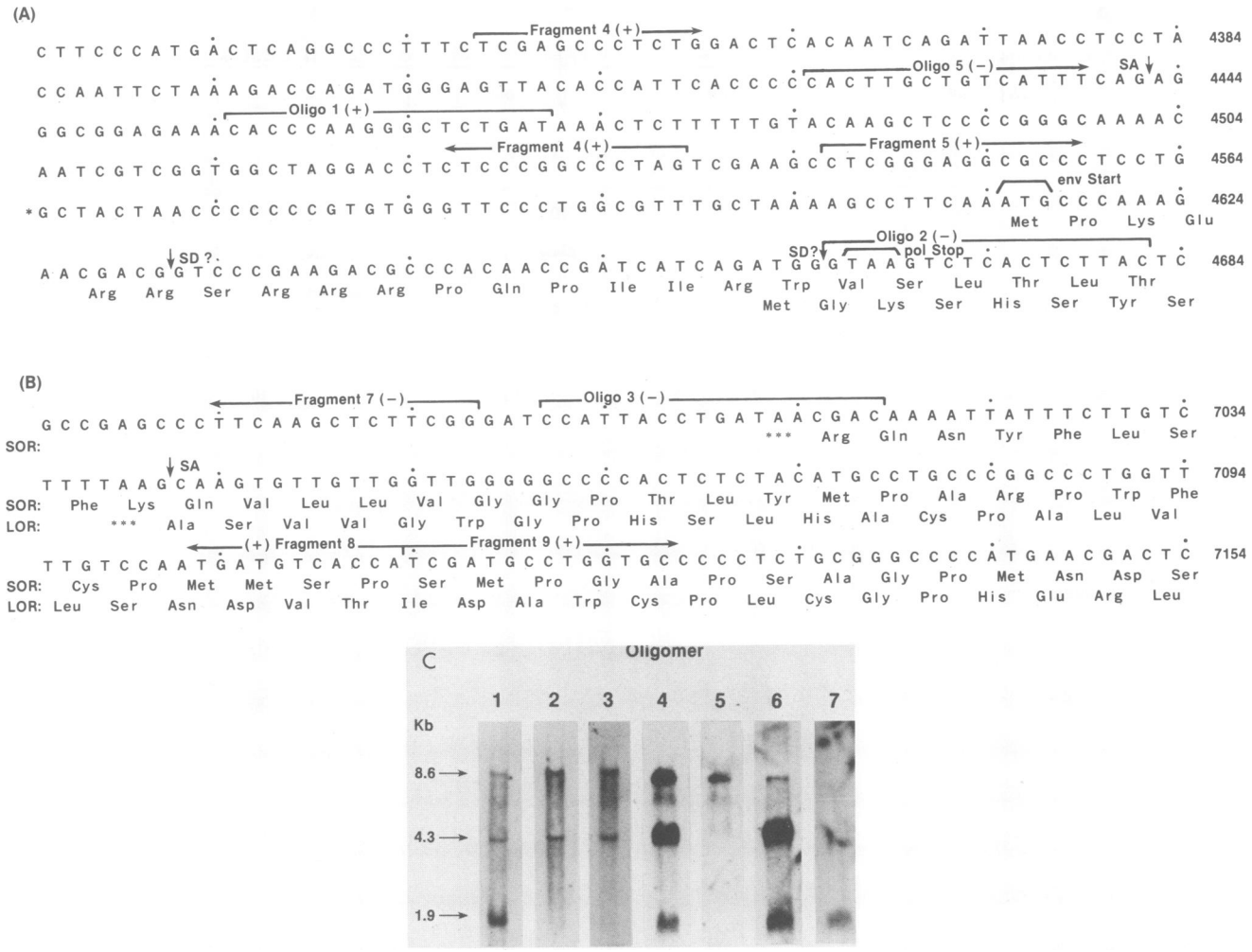

FIG. 2. Splicing of the X-region mRNA in BLV-infected bat cells. (A) Nucleotide sequence from 4325 to 4684 (cap site is position 1) of the Belgian tumor-derived clone. The boundaries of the two DNA fragments capable of hybridizing to the 1.9-kb RNA (fragments 4 and 5) are indicated, as are the boundaries of oligomer 1, which also hybridizes, and oligomers 2 and 5, which do not. + and −, Positive and negative hybridization. The deduced splice acceptor (33) upstream of oligomer 1 is indicated, as are possible splice donors (33) downstream of the *env* initiator at 4615 to 4617. In the oligomer 2 region, the FLK clone derived by Jim Casey differs at one position from the tumor-derived clone (Rice and Stephens, unpublished results); oligomer 2 was made complementary to the FLK clone. (B) Nucleotide sequence from 6975 to 7154 within the X region. The 5′ boundary of fragment 7, which does not hybridize to the 1.9-kb RNA, is the same (6614) as the 5′ boundary of fragment 8, which does hybridize. Their 3′ boundaries are indicated in the figure, as are the boundaries of oligomer 3, which does not hybridize to the 1.9-kb RNA. The only possible splice acceptor between oligomer 3 and the 3′ boundary of fragment 8 is indicated. (C) Hybridization of oligonucleotide probes to Northern blots of RNA from BLV-infected bat cells. Oligomers 1, 2, 3, and 5 are described in panels A and B, above. Oligomer 4 is complementary to the extreme 3′ end of viral RNA and is included as a positive control to show all the major hybridizing species. Oligomer 6 is complementary to bases 86 to 94 and 4443 to 4453 of genomic RNA, and oligomer 7 is complementary to bases 4657 to 4665 and 7042 to 7051 of genomic RNA. The weak hybridization of oligomer 6 to genomic RNA was unexpected and may reflect its high G+C content (65%).

The splice acceptor for both the *env* mRNA and X mRNA must therefore lie between these two regions. The only possible acceptor which obeys the consensus rules compiled by Mount (33) is at 4443, 172 bases 5′ of the initiation codon for the *env* gene. In accordance with this prediction, oligomer 6 was able to hybridize with both the *env* and X mRNAs (Fig. 2C). Oligomer 6 is complementary to a 20-base sequence predicted to occur in spliced mRNA but absent from genomic RNA; it spans the predicted splice junction between the 5′ end of viral RNA (bases 86 to 94) and the splice acceptor at 4443 (bases 4443 to 4453).

Oligomer 2 was designed to illuminate the position of the splice donor, which must be located within fragment 5. Oligomer 2 is complementary to bases 52 to 68 of the *env* gene (positions 4666 to 4682 of the BLV genome) and did not hybridize to the 1.9-kb RNA (Fig. 2A and C). If the X protein(s) begins with a methionine supplied by *env*, then the splice donor must be located between 4618 (base 4 of *env*) and about 4680. There is only one good match to the consensus splice donor $^{C}_{A}$AG/GT$^{A}_{G}$AGT (33) within this region; it is TGG/GTAAGT located at 4665.

The splice acceptor in the X region can also be predicted. Since fragment 7 (6614 to 6997) did not hybridize with the 1.9-kb RNA but fragment 8 (6614 to 7113) did, the splice

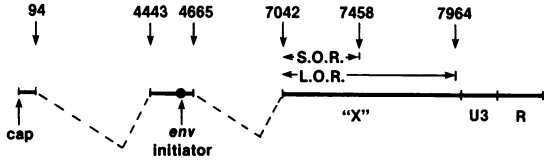

FIG. 3. Deduced structure of the doubly spliced BLV X-region mRNA.

acceptor must be located between about 6997 and 7113. Oligomer 3 (7001 to 7018) also did not hybridize to the 1.9-kb RNA (Fig. 2B and C). There is only one good match to the consensus acceptor sequence in the region defined by these results; it is located at 7042 (Fig. 2B). In accordance with these predictions, oligomer 7, which spans the predicted splice junction (bases 4656 to 4665 and 7042 to 7051), was able to hybridize only with X mRNA (Fig. 2C). Thus, the structure of the 1.9-kb X mRNA is as diagrammed in Fig. 3.

Structure of X mRNA predicts translation of the short open reading frame. The 5'-most methionine codon in the X mRNA is the envelope initiator at 4615 to 4617 (Fig. 2A). If translation begins there, the resulting protein will contain 17 amino acids of the *env* signal peptide. These 17 do not include the very hydrophobic portion of the signal peptide (39), and unlike the *env* polyprotein, therefore, the 1.9-kb RNA translation product would not be membrane directed. The remainder of the protein is encoded in the X region beginning with the splice acceptor at 7042. Given the reading frame of the *env* leader and the locations of the splice donor and acceptor, this protein must be translated from the short open reading frame (Fig. 2A and B). It is predicted to contain 156 amino acids and have a molecular weight of 16,575.

Detection of the X-*sor* protein. To search for the predicted X-*sor* protein, antibodies were raised to two synthetic peptides derived from the translated sequence (see Materials and Methods and Fig. 7). The antisera were then used in immune-precipitation experiments with lysates of [^{35}S]methionine- or [^{35}S]cysteine-labeled BLV-infected FLK cells. Both antisera precipitated a protein of approximately 19,000 molecular weight (Fig. 4A, lanes 2 and 6). This protein was not observed when preimmune serum was used (lanes 1 and 5). Precipitation was prevented in the presence of excess homologous peptide (lanes 3 and 7), but excess heterologous peptide had no effect (lanes 4 and 8). On the basis of this specificity and positive results with antisera to two independent peptides, we conclude that p19 is the product of BLV X-*sor*. As expected if p19 is the X-*sor* product, it was also detectable in BLV-infected bat cells but not in uninfected bat cells (Fig. 4B).

Antiserum I also precipitated a protein of about 68,000 molecular weight which was not seen with preimmune serum and whose binding was inhibited by homologous but not by heterologous peptide. The identity of this protein is not known, but since it was not recognized by antiserum II, we think it unlikely that it is significantly related to p19. It is well known that antisera to synthetic peptides may cross-react with a small number of other proteins, presumably by virtue of their sequence homology to the peptide (35).

X-*sor* protein is phosphorylated. To determine whether p19 is a phosphoprotein, FLK cells were grown in the presence of $^{32}P_i$ and lysates were immune precipitated with each of the two peptide antisera. In each case, a ^{32}P-labeled doublet was observed, with the individual proteins migrating with apparent molecular weights of about 19,000 and 20,000 (Fig. 5, lanes 2 and 5). These proteins were not precipitated by preimmune serum (lanes 1 and 4), and precipitation by immune serum was inhibited by homologous peptide (lanes 3 and 6). The 20,000-molecular-weight member of the doublet was present in small amounts in lysates of ^{35}S-labeled cells (Fig. 4 and 6) but was easily detectable upon ^{32}P labeling, perhaps indicating that it is very highly phosphorylated. Since sodium vanadate (a phosphatase inhibitor) was included during the ^{32}P labeling but not during the ^{35}S labeling, phosphatase action may have been significantly higher in the latter, resulting in the preferential loss of p20.

To determine the identity of the phosphorylated amino acid, immune precipitates of ^{32}P-labeled lysates were applied to a preparative polyacrylamide gel, and the 19,000- to 20,000-molecular-weight doublet was excised. Protein was soaked out of the gel slices, hydrolyzed in 4 N HCl, and analyzed on a thin-layer chromatography plate (in the presence of phosphoamino acid standards) by electrophoresis in one direction and chromatography in the second. Radioactivity appeared entirely in the phosphoserine spot, with none detectable in phosphothreonine or phosphotyrosines (data not shown).

X-*sor* protein is predominantly nuclear. To investigate the subcellular localization of p19, ^{35}S-labeled lysates of FLK cells were immune precipitated after separation into nuclear and cytoplasmic fractions. Briefly, cells were lysed in TNT (20 mM Tris [pH 7.5], 200 mM sodium chloride, 1% Triton X-100), and a crude nuclear pellet was collected by centrifugation at 600 × *g* for 15 min. This pellet was resuspended in TNT and recentrifuged to yield a nuclear-wash fraction and the final nuclear pellet. The first supernatant was centrifuged again at 600 × *g* for 15 min to remove any remaining nuclei and then centrifuged at 10,000 × *g* for 10 min. The supernatant was the final cytoplasmic (including membrane) fraction. SDS was added to each fraction to a final concentration of 0.25%, and insoluble material was removed by centrifugation at 10,000 × *g* for 10 min. The validity of the fractionation was monitored in two ways. First, the location of the viral envelope glycoprotein was determined in the ^{35}S-labeled fractions, and second, an additional aliquot of cells was incubated in [^{3}H]thymidine and fractionated as described above.

Immune precipitation of the ^{35}S-labeled fractions revealed that p19 was predominantly located in the nuclear fraction (Fig. 6, lanes 2 and 4), although a small amount was detectable in the cytoplasm, particularly with antiserum II (lane 4). In addition, both members of the p19 doublet were clearly visible in the nuclear fraction. As in previous experiments, the p19 doublet was not detected by preimmune antiserum (lane 1) and was inhibited with excess homologous peptide (lanes 3 and 5).

Localization of the BLV envelope glycoprotein gp60 was monitored by use of α-gp60 serum (46). As expected, gp60 was predominantly located in the cytoplasmic fraction (Fig. 6, lane 6), although a small amount sedimented with the nuclei. In contrast, 98% of the acid-precipitable [^{3}H]thymidine radioactivity was located in the nuclear fraction (Table 1). We conclude that the fractionation procedure yields a good separation of nuclei and cytoplasm, and we can therefore have confidence in the conclusion that p19 is a predominantly nuclear protein. It is likely that under our conditions for the isolation of nuclei, the nuclear envelope was dissolved and soluble proteins were extracted (10, 53). We can further conclude, therefore, that most of p19 is bound to a nuclear element or elements.

We also assayed virus for the presence of p19. The

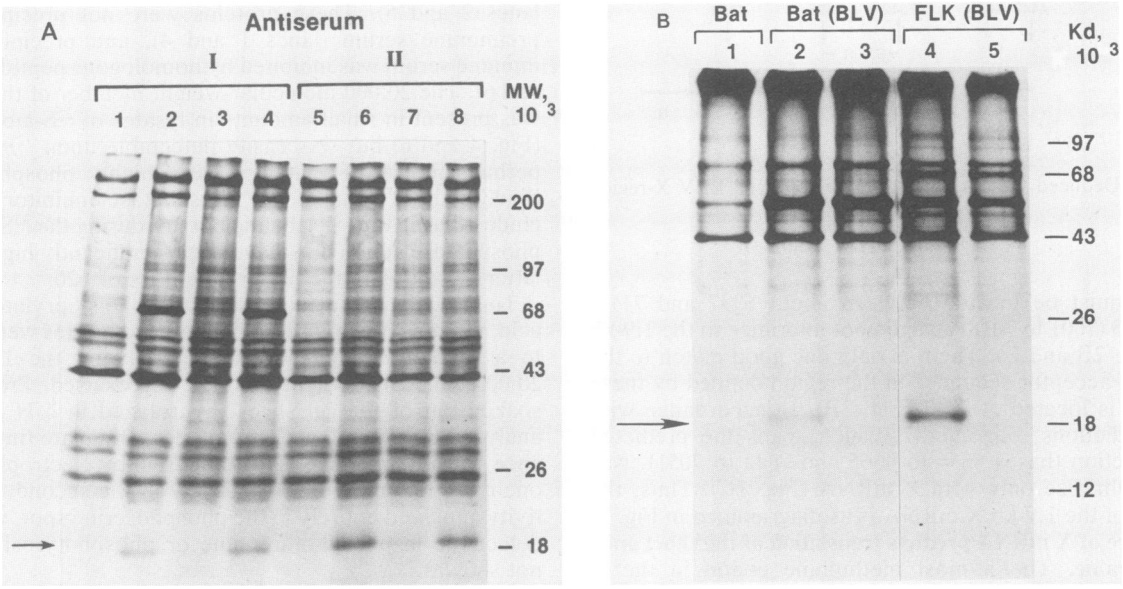

FIG. 4. Detection of the BLV X-*sor* protein. (A) FLK cells (a confluent T150 flask) were labeled with [^{35}S]methionine and [^{35}S]cysteine, each at 100 μCi/ml for 4 h. Immune precipitates were prepared as described in Materials and Methods and were fractionated on a 10 to 20% SDS-polyacrylamide gel. Lanes: 1 and 5, preimmune antiserum; 2, antiserum I; 3, antiserum I plus competing peptide I; 4, antiserum I plus competing peptide II; 6, antiserum II; 7, antiserum II plus competing peptide II; 8, antiserum plus competing peptide I. (B) Cells were labeled in 25 μCi of [^{35}S]methionine per ml for 2 h, and lysates were immune precipitated and fractionated as described above. Lanes: 1, 2, and 4, antiserum I; 3 and 5, antiserum I plus competing peptide I.

medium was collected after a 4-h incubation in [^{35}S]methionine and [^{35}S]cysteine, clarified, and centrifuged at 100,000 × *g* for 1 h. The viral pellet was lysed and subjected to immune precipitation. The presence of viral proteins was confirmed by the use of αgp60 and αp24, both of which resulted in very dark bands on the autoradiograph, but no p19 was detected even after long exposure (data not shown).

DISCUSSION

Previous work has demonstrated the existence of a doubly spliced BLV mRNA of 1.8 to 2.1 kb in virus-infected cells (30, 44) and of a 38-kilodalton protein which is the product of the X-region long open reading frame (43). In the present study, we confirmed the existence of the X-region mRNA, deduced the location of the splice sites, and predicted and

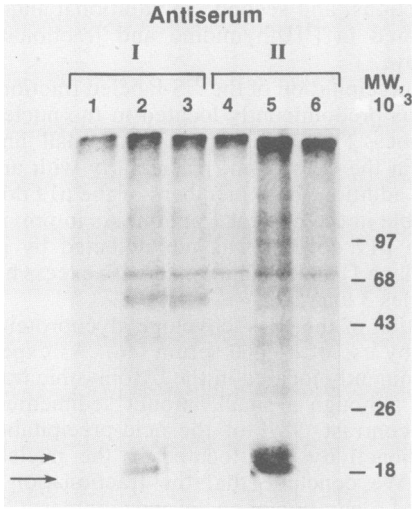

FIG. 5. X-*sor* protein is phosphorylated. FLK cells (a confluent T150 flask) were grown in ^{32}Pi as described in Materials and Methods. Lysates were immune precipitated and fractionated on a 10 to 20% SDS-polyacrylamide gel. The gel was dried and exposed to X-ray film at −70°C for 2 days with an intensifying screen. Lanes: 1 and 4, preimmune serum; 2, antiserum 1; 3, antiserum 1 plus competing peptide 1; 5, antiserum 2; 6, antiserum 2 plus competing peptide 2.

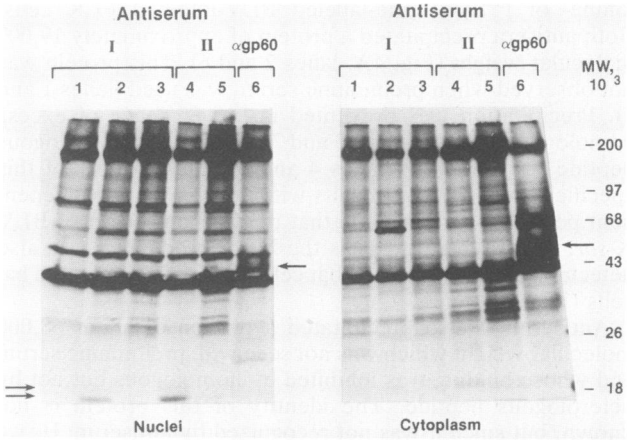

FIG. 6. Subcellular localization of the X-*sor* phosphoprotein p19. FLK cells were labeled with 100 μCi of [^{35}S]methionine per ml for 5 h and then lysed and fractionated as described in the text. Lanes for each of the nuclear and cytoplasmic fractions: 1, preimmune serum; 2, antiserum I; 3, antiserum I plus competing peptide I; 4, antiserum II; 5, antiserum II plus competing peptide II; 6, αgp60. The three additional subcellular fractions (nuclei$_2$, nuclear wash, and 10,000 × *g* pellet [for definitions, see Table 1, footnote *a*]) were assayed in the same way. No p19 was detected in any of the three, while a trace of gp 60 was seen in the nuclear wash (data not shown).

TABLE 1. Distribution of [^{3}H]thymidine after subcellular fractionation[a]

Fraction	[^{3}H]thymidine distribution			
	cpm in 10 μl	Total vol (ml)	Total cpm (10^6)	% Total cpm
Nuclei	38,000	2.6	9.9	98
$Nuclei_2$	2,900	1.0	0.29	
Nuclear wash	660	2.2	0.15	1.4
10,000 × *g* pellet	390	1.0	0.04	0.4
Cytoplasm	160	2.4	0.04	0.4

[a] A subconfluent T150 flask of FLK cells was incubated overnight in complete medium plus 10% dialyzed calf serum plus 5 μCi of [^{3}H]thymidine (51 Ci/mmol) per ml. Cells were lysed and fractionated as described in the text. $Nuclei_2$ is the pellet from the second centrifugation at 600 × *g*, and cytoplasm is the supernatant from the centrifugation at 10,000 × *g* centrifugation. The number of counts per minute insoluble in 10% trichloroacetic acid was determined for 10 μl of each fraction.

then demonstrated the occurrence of a new protein translated from this RNA. This X-*sor* protein migrated as a doublet of 19,000 and 20,000 molecular weight in SDS-polyacrylamide gel electrophoresis. It is a nuclear phosphoprotein.

If translation of the X mRNA begins at its 5′-most initiation codon (which in BLV is the *env* gene initiator), then the X-*sor* protein will have the primary structure indicated in Fig. 7. A total of 17 residues will be translated from the *env* gene, and 139 will be translated from X. Initiation at a similarly placed AUG in HTLV-I would produce a protein containing 20 pre-*env* residues and 169 residues from X (Fig.

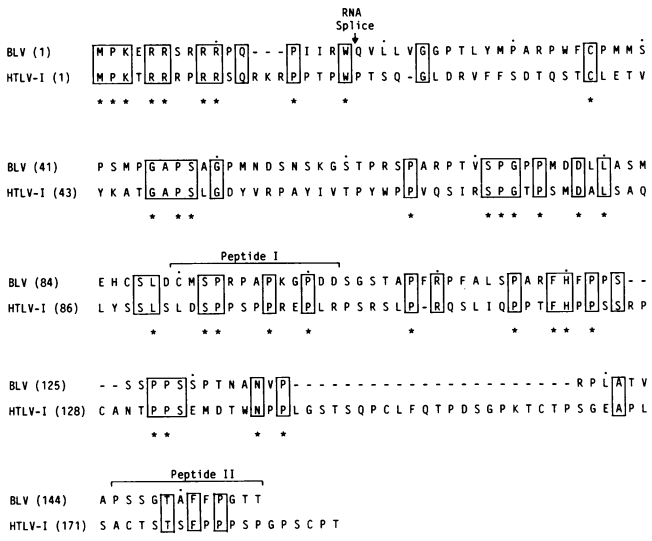

FIG. 7. Predicted amino acid sequence of the BLV X-*sor* protein. All identities between BLV and HTLV-I (47) are boxed. Common residues which are also found in the predicted HTLV-II protein (50) are indicated by an asterisk. There is a dot over every tenth BLV amino acid. The location of the splice in the mRNAs from which these proteins are presumably translated is indicated; upstream of the splice site, the RNA is transcribed from the *env* gene; downstream, it is transcribed from the X region. The locations of peptides I and II are also shown. The BLV sequence shown is that of the Belgian tumor-derived clone (39). A clone derived from the FLK virus-producing cell line by Jim Casey contains several single-base changes (Rice and Stephens, unpublished results). At residue 146, the FLK clone encodes leucine instead of serine; peptide II corresponds to the FLK sequence.

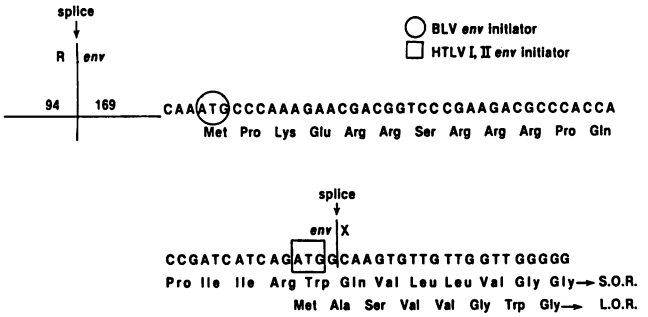

FIG. 8. Structure and partial sequence (39) of the BLV X mRNA. Initiation at the first AUG codon would result in translation of the *sor* protein; initiation at the second AUG would result in translation of the *lor* protein. The arrangement of AUG codons and reading frames is identical in BLV, HTLV-I (47), and HTLV-II (52), although in HTLV-I and -II, the second AUG and not the first is the initiator for the envelope protein.

7). The HTLV-I protein (p27) has been detected both in virus-infected cells (26) and after in vitro translation of X-region mRNA (34). Mutational analysis has revealed that it is indeed initiated at the 5′-most AUG (34) and that it must have the structure indicated in Fig. 7. Until very recently, we had no evidence that BLV translation initiates at the first AUG, but we have now raised an antiserum against a peptide representing the extreme N terminus (residues 3 to 16) of the predicted X-*sor* protein. Since this serum recognized the 19,000- to 20,000-molecular-weight doublet (data not shown), translation must initiate at the first AUG.

The next in-frame AUG codon in the HTLV-I sequence does not occur for more than 230 bases. Nevertheless, translation occasionally initiates there to produce p21, a phosphoprotein of about 12,000 molecular weight, which migrates anomalously in SDS gels (26, 34). In BLV, the second in-frame AUG codon occurs 84 bases downstream from the first, and initiation there would result in a protein of 128 residues. Such a protein should be detectable with both of our antisera, but to date we have seen only the 19,000- to 20,000-molecular-weight doublet. The second in-frame AUG does act as an initiator during in vitro translation of unfractionated viral RNA (58).

Of the first 10 residues of the X-*sor* protein, 7 are charged, but the N terminus is the only such highly charged region in the molecule. Downstream, charged residues occur at about half the average protein rate and they are separated by long uncharged regions. In BLV there are uncharged stretches of 14, 17, 18, and 21 residues, which together account for 45% of the molecule. The situation is similar in the HTLV-I protein. Both proteins are also very high in proline (21%) and serine (14 and 15%). Overall, 46 of the 156 residues of the BLV protein are also found in the HTLV-I protein (29% identity) in an alignment requiring five gaps (Fig. 7). Thus, their X-*sor* proteins are less homologous than any of the viral *gag*, *pol*, and *env* products, with the possible exception of the envelope glycoprotein (38, 39).

In both BLV and HTLV, an X protein encoded by the long open reading frame has been identified (29, 43, 51). How is it translated? Inspection of the deduced structure of BLV X mRNA (Fig. 8) reveals that both the X-*sor* and X-*lor* proteins could be translated from the same mRNA. As we have seen, if translation in BLV initiates at the *env* gene AUG, the X-*sor* protein is produced. But the *env* gene initiator is in a suboptimal context for initiation (27) and may therefore be bypassed at rather high frequency. If translation initiates at

the next AUG codon (located at 4662 to 4664 in Fig. 2A), the X-*lor* protein could be produced. The same arrangement of AUG codons and reading frames exists in HTLV-I and -II, leading to the same prediction; i.e., the X-*lor* and X-*sor* proteins are translated from the same mRNA and result from initiation at different AUG codons. Recently, Nagashima et al. (34) elegantly verified this prediction for the HTLV-I X proteins. Other precedents for this arrangement have been established in the human adenoviruses 5 and 12 (7), in Sendai virus (13, 18), in influenza B (49), in measles virus (5), in reovirus (16), and possibly within the *src* region of Rous sarcoma virus (32) as well.

The prediction of a single mRNA for translation of both BLV proteins is based on the assumption that the splice sites at 4665 and 7042 (Fig. 3) are used for all X-mRNA molecules. We cannot rule out the possibility that additional RNA species exist which use slightly different sites and from which only one of the proteins can be translated. The splice sites used to generate such RNAs would have to be very poor matches to the consensus sequences, however. The only conclusive way to demonstrate the translation of the two proteins from a single RNA is to insert a cDNA clone into a vector suitable for transcription by the SP6 polymerase. The resulting homogeneous RNA population could then be used in an in vitro translation system, and the number of proteins could be determined. This has been accomplished for HTLV-I (34), and on the basis of their overall relatedness, we assume a similar situation for BLV. Nevertheless, possible heterogeneity in splice sites cannot be dismissed, for there is some evidence that the position of the acceptor upstream of the *env* initiation codon varies. For example, oligomer 5, which lies upstream of the major splice acceptor at 4443, was able to hybridize very weakly with an RNA slightly larger than most of the *env* mRNA (Fig. 2C). This may indicate that an alternative splice acceptor lies somewhat upstream of 4443. Consistent with this possibility, a BLV X-region cDNA clone has recently been described in which an upstream acceptor must have been used (42). Significantly, the splice sites we established which determine the synthesis of both X proteins, the donor at 4665 and the acceptor at 7042, were also found in the cDNA clone (42).

The function of the X-*sor* protein is unknown. It will be of obvious interest to determine the nuclear component to which it binds, as well as its possible affinity for the X-*lor* protein. The present findings should also prompt an examination of other retroviral sequences for the occurrence of overlapping reading frames.

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LITERATURE CITED

1. **Aldovini, A., A. De Rossi, M. B. Feinberg, F. Wong-Staal, and G. Franchini.** 1986. Molecular analysis of a deletion mutant provirus of type I human T-cell lymphotropic virus: evidence for a doubly spliced x-lor mRNA. Proc. Natl. Acad. Sci. USA **83**:38–42.
2. **Aviv, H., and P. Leder.** 1972. Purification of biologically active globin messenger RNA by chromatography on oligothymidylic acid-cellulose. Proc. Natl. Acad. Sci. USA **69**:1408–1412.
3. **Bailey, J. M., and N. Davidson.** 1976. Methylmercury as a reversible denaturing agent for agarose gel electrophoresis. Anal. Biochem. **70**:75–85.
4. **Barany, G., and R. B. Merrifield.** 1980. Solid phase peptide synthesis, p. 1–284. *In* E. Gross and J. Meienhofer (ed.), The peptides, vol. 2. Academic Press, Inc., New York.
5. **Bellini, W. J., G. Englund, S. Rozenblatt, H. Arnheiter, and C. D. Richardson.** 1985. Measles virus *P* gene codes for two proteins. J. Virol. **53**:908–919.
6. **Bender, W., Y. H. Chien, S. Chattopadhyay, P. K. Vogt, M. B. Gardner, and N. Davidson.** 1978. High-molecular-weight RNAs of AKR, NZB, and wild mouse viruses and avian reticuloendotheliosis virus all have similar dimer structure. J. Virol. **25**:888–896.
7. **Bos, J. L., L. J. Polder, R. Bernards, P. I. Schrier, P. J. van den Elsen, A. J. van der Eb, and H. van Ormondt.** 1981. The 2.2 kb E1b mRNA of human Ad12 and Ad5 codes for two tumor antigens starting at different AUG triplets. Cell **27**:121–131.
8. **Burny, A., C. Bruck, H. Chantrene, Y. Cleuter, D. Dekegel, J. Ghysdael, R. Kettmann, M. Lellercq, J. Leunen, M. Mammerickx, and D. Portetelle.** 1980. Bovine leukemia virus: molecular biology and epidemiology, p. 231–289. *In* G. Klein (ed.), Viral oncology. Raven Press, New York.
9. **Cann, A. J., J. D. Rosenblatt, W. Wachsman, N. P. Shah, and I. S. Y. Chen.** 1985. Identification of the gene responsible for human T-cell leukemia virus transcriptional regulation. Nature (London) **318**:571–574.
10. **Capco, D. G., K. M. Wan, and S. Penman.** 1982. The nuclear matrix: three-dimensional architecture and protein composition. Cell **29**:847–858.
11. **Copeland, T. D., M. A. Morgan, and S. Oroszlan.** 1983. Complete amino acid sequence of the nucleic acid binding protein of bovine leukemia virus. FEBS Lett. **156**:37–40.
12. **Copeland, T. D., S. Oroszlan, V. S. Kalyanaraman, M. G. Sarngadharan, and R. C. Gallo.** 1983. Complete amino acid sequence of human T-cell leukemia virus structural protein p15. FEBS Lett. **162**:390–395.
13. **Curran, J. A., C. Richardson, and D. Kolakofsky.** 1986. Ribosomal initiation at alternate AUGs on the Sendai virus P/C mRNA. J. Virol. **57**:684–687.
14. **Deeley, R. G., J. I. Gordon, A. T. H. Burns, K. P. Mullinix, M. Binastein, and R. F. Goldberger.** 1977. Primary activation of vitellogenin gene in rooster. J. Biol. Chem. **252**:8310–8319.
15. **Deschamps, J., R. Kettmann, and A. Burny.** 1981. Experiments with cloned complete tumor-derived bovine leukemia virus information prove that the virus is totally exogenous to its target animal species. J. Virol. **40**:605–609.
16. **Ernst, H., and A. J. Shatkin.** 1985. Reovirus hemagglutinin mRNA codes for two polypeptides in overlapping reading frames. Proc. Natl. Acad. Sci. USA **82**:48–52.
17. **Felber, B. K., H. Paskalis, C. Kleinman-Ewing, F. Wong-Staal, and G. N. Pavlakis.** 1985. The pX protein of HTLV-I is a transcriptional activator of its long terminal repeats. Science **229**:675–679.
18. **Giorgi, C., B. M. Blumberg, and D. Kolakofsky.** 1983. Sendai virus contains overlapping genes expressed from a single mRNA. Cell **35**:829–836.
19. **Goldberg, D. A.** 1980. Isolation and partial characterization of the *Drosophila* alcohol dehydrogenase gene. Proc. Natl. Acad. Sci. USA **77**:5794–5798.
20. **Gonda, M. A., N. R. Rice, and R. V. Gilden.** 1980. Avian reticuloendotheliosis virus: characterization of the high-molecular-weight viral RNA in transforming and helper virus populations. J. Virol. **34**:743–751.
21. **Graves, D. C., and J. F. Ferrer.** 1975. In vitro transmission and propagation of the bovine leukemia virus in monolayer cell cultures. Cancer Res. **36**:4152–4159.
22. **Grégoire, D., D. Couez, J. Deschamps, S. Heuertz, M.-C. Hors-Cayla, J. Szpirer, C. Szpirer, A. Burny, G. Huez, and R. Kettmann.** 1984. Different bovine leukemia virus-induced tu-

mors harbor the provirus in different chromosomes. J. Virol. **50**:275–279.
23. **Kettmann, R., Y. Cleuter, D. Gregoire, and A. Burny.** 1985. Role of the 3′ long open reading frame region of bovine leukemia virus in the maintenance of cell transformation. J. Virol. **54**:899–901.
24. **Kettmann, R., Y. Cleuter, M. Mammerickx, M. Meunier-Rotival, G. Bernardi, A. Burny, and H. Chantrenne.** 1980. Genomic integration of bovine leukemia provirus: comparison of persistent lymphocytosis with lymph-node tumor form of enzootic bovine leukosis. Proc. Natl. Acad. Sci. USA **77**:2577–2581.
25. **Kettmann, R., J. Deschamps, Y. Cleuter, D. Couez, A. Burny, and G. Marbaix.** 1982. Leukemogenesis by bovine leukemia virus: proviral DNA integration and lack of RNA expression of viral long terminal repeat and 3′ proximate cellular sequences. Proc. Natl. Acad. Sci. USA **79**:2465–2469.
26. **Kiyokawa, T., M. Seiki, S. Iwashita, K. Imagawa, F. Shimizu, and M. Yoshida.** 1985. $p27^{x\text{-}III}$ and $p21^{x\text{-}III}$, proteins encoded by the pX sequence of human T-cell leukemia virus type I. Proc. Natl. Acad. Sci. USA **82**:8359–8363.
27. **Kozak, M.** 1986. Point mutations define a sequence flanking the AUG initiator codon that modulates translation by eukaryotic ribosomes. Cell **44**:283–292.
28. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **277**:680–685.
29. **Lee, T. H., J. E. Coligan, J. G. Sodroski, W. A. Haseltine, S. Z. Salahuddin, F. Wong-Staal, R. C. Gallo, and M. Essex.** 1984. Antigens encoded by the 3′ terminal region of human T-cell leukemia virus: evidence for a functional gene. Science **226**:57–61.
30. **Mamoun, R. Z., T. Astier-Gin, R. Kettmann, J. Deschamps, N. Rebeyrotte, and B. J. Guillemain.** 1985. The pX region of the bovine leukemia virus is transcribed as a 2.1-kilobase mRNA. J. Virol. **54**:625–629.
31. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1982. Molecular cloning: a laboratory manual, p. 202–203. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
32. **Mardon, G., and H. E. Varmus.** 1983. Frameshift and intragenic suppressor mutations in a Rous sarcoma provirus suggest *src* encodes two proteins. Cell **32**:871–879.
33. **Mount, S. M.** 1982. A catalogue of splice junction sequences. Nucleic Acids Res. **10**:459–472.
34. **Nagashima, K., M. Yoshida, and M. Seiki.** 1986. A single species of *pX* mRNA of human T-cell leukemia virus type I encodes *trans*-activator $p40^{x}$ and two other phosphoproteins. J. Virol. **60**:394–399.
35. **Nigg, E. A., G. Walter, and S. J. Singer.** 1982. On the nature of cross-reactions observed with antibodies directed to defined epitopes. Proc. Natl. Acad. Sci. USA **79**:5939–5943.
36. **Oroszlan, S., M. G. Sarngadharan, T. D. Copeland, V. S. Kalyanaraman, R. V. Gilden, and R. C. Gallo.** 1982. Primary structure analysis of the major internal protein p24 of human type C T-cell leukemia virus. Proc. Natl. Acad. Sci. USA **79**:1291–1294.
37. **Rice, N. R., T. D. Copeland, S. Simek, S. Oroszlan, and R. V. Gilden.** 1986. Detection and characterization of the protein encoded by the v-*rel* oncogene. Virology **149**:217–229.
38. **Rice, N. R., R. M. Stephens, A. Burny, and R. V. Gilden.** 1985. The *gag* and *pol* genes of bovine leukemia virus: nucleotide sequence and analysis. Virology **142**:357–377.
39. **Rice, N. R., R. M. Stephens, D. Couez, J. Deschamps, R. Kettmann, A. Burny, and R. V. Gilden.** 1984. The nucleotide sequence of the *env* gene and post-*env* region of bovine leukemia virus. Virology **138**:82–93.
40. **Rice, N. R., R. M. Stephens, and R. V. Gilden.** 1987. Sequence analysis of the bovine leukemia virus genome, p. 115–144. *In* A. Burny and M. Mammerickx (ed.), Enzootic bovine leukosis and bovine leukemia virus. Martinus Nijhoff, Leiden, The Netherlands.
41. **Rigby, P. W. J., M. Dieckmann, C. Rhodes, and P. Berg.** 1977. Labeling of deoxyribonucleic acid to high specific activity in vitro by nick translation with DNA polymerase I. J. Mol. Biol. **113**:237–251.
42. **Rosen, C. A., J. G. Sodroski, L. Willems, R. Kettmann, K. Campbell, R. Zaya, A. Burny, and W. A. Haseltine.** 1986. The 3′ region of bovine leukemia virus genome encodes a trans-activator protein. EMBO J. **5**:2585–2589.
43. **Sagata, N., J. Tsuzuku-Kawamura, M. Nagayoshi-Aida, F. Shimizu, K.-I. Imagawa, and Y. Ikawa.** 1985. Identification and some biochemical properties of the major X_{BL} gene product of bovine leukemia virus. Proc. Natl. Acad. Sci. USA **82**:7879–7883.
44. **Sagata, N., T. Yasunaga, and Y. Ikawa.** 1985. Two distinct polypeptides may be translated from a single spliced mRNA of the X genes of human T-cell leukemia and bovine leukemia viruses. FEBS Letts. **192**:37–42.
45. **Sagata, N., T. Yasunaga, J. Tsuzuku-Kawamura, K. Ohishi, Y. Ogawa, and Y. Ikawa.** 1985. Complete nucleotide sequence of the genome of bovine leukemia virus: its evolutionary relationship to other retroviruses. Proc. Natl. Acad. Sci. USA **82**:677–682.
46. **Schultz, A. M., T. D. Copeland, and S. Oroszlan.** 1984. The envelope proteins of bovine leukemia virus: purification and sequence analysis. Virology **135**:417–427.
47. **Seiki, M., S. Hattori, Y. Hirayama, and M. Yoshida.** 1983. Human adult T-cell leukemia virus: complete nucleotide sequence of the provirus genome integrated in leukemia cell DNA. Proc. Natl. Acad. Sci. USA **80**:3618–3622.
48. **Seiki, M., A. Hikikoshi, T. Taniguchi, and M. Yoshida.** 1985. Expression of the pX gene of HTLV-I: general splicing mechanism in the HTLV family. Science **228**:1532–1534.
49. **Shaw, M. W., P. W. Choppin, and R. A. Lamb.** 1983. A previously unrecognized influenza B virus glycoprotein from a bicistronic mRNA that also encodes the viral neuraminidase. Proc. Natl. Acad. Sci. USA **80**:4879–4883.
50. **Shimotohno, K., Y. Takahashi, N. Shimizu, T. Gojobori, D. W. Golde, I. S. Y. Chen, M. Miwa, and T. Sugimura.** 1985. Complete nucleotide sequence of an infectious clone of human T-cell leukemia virus type II: an open reading frame for the protease gene. Proc. Natl. Acad. Sci. USA **82**:3101–3105.
51. **Slamon, D. J., K. Shimotohno, M. J. Cline, D. W. Golde, and I. S. Y. Chen.** 1984. Identification of the putative transforming protein of the human T-cell leukemia viruses HTLV-I and HTLV-II. Science **226**:61–65.
52. **Sodroski, J., C. Rosen, W. C. Goh, and W. Haseltine.** 1985. A transcriptional activator protein encoded by the x-*lor* region of the human T-cell leukemia virus. Science **228**:1430–1434.
53. **Staufenbiel, M., and W. Deppert.** 1982. Intermediate filament systems are collapsed onto the nuclear surface after isolation of nuclei from tissue culture cells. Exp. Cell. Res. **138**:207–214.
54. **Tager, H. S.** 1976. Coupling of peptides with difluorodinitrobenzene. Anal. Biochem. **71**:367–375.
55. **Thomas, P.** 1980. Hybridization of denatured RNA and small DNA fragments transferred to nitrocellulose. Proc. Natl. Acad. Sci. USA **77**:5201–5205.
56. **Vander Maaten, M. J., and J. M. Miller.** 1976. Replication of bovine leukemia virus in monolayer cell cultures. Bibl. Haematol. **43**:360–362.
57. **Wachsman, W., D. W. Golde, P. A. Temple, E. C. Orr, S. C. Clark, and I. S. Y. Chen.** 1985. HTLV x-gene product: requirement for the *env* methionine initiation codon. Science **228**:1534–1537.
58. **Yoshinaka, Y., and S. Oroszlan.** 1985. Bovine leukemia virus post-envelope gene coded protein: evidence for expression in natural infection. Biochem. Biophys. Res. Commun. **131**:347–354.