

Identification of the Bovine Leukemia Virus Transactivating Protein (p34^x)

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A. Introduction

Enzootic bovine leukosis (EBL) has been recognized as a neoplasm of infectious origin for half a century. The agent, bovine leukemia virus (BLV), is a retrovirus discovered in 1969 in short-term cultures of peripheral lymphocytes from animals with persistent lymphocytosis, a benign response to BLV infection. A virus distantly related to BLV was more recently identified as the etiological agent in the vast majority of cases of adult T cell leukemia and named for that reason human T-lymphotropic virus I (HTLV-I) [20]. The pathologies of BLV- and HTLV-I-induced diseases are notably similar, namely absence of chronic viremia, a long latency period, and lack of preferred integration sites in tumors. A second human virus, called HTLV-II, was identified in the Mo T cell line, derived in 1976 from the spleen of a patient with T cell-variant hairy cell leukemia [2, 10]. Other isolates of HTLV-I and -II have since been obtained around the world. Both viruses not only transform normal T-lymphocytes but might also very well be involved in a number of degenerative diseases of the nervous system.

The genomes of HTLV-I and -II show a nucleic acid sequence homology of about 60%. Relatedness between HTLV-I and BLV varies from 30% to more than 50%,

according to the genes under consideration [22, 23, 27]. Sequence similarities between the virion proteins of BLV, HTLV-I, and HTLV-II have also been reported [3, 18]. Moreover, the three viruses have in common the fact that they contain several overlapping open reading frames located between the *env* gene and the 3' long terminal repeat (LTR). In these three cases, proteins coded by that region of the genome have been shown to transactivate the LTR of the provirus, hence their generic name of *tat* proteins (for transactivation of transcription) [13, 31, 34].

As a 34- to 38-kD protein predominantly located in the nucleus of the infected cell [28], the putative *tat* BLV product p34^x [26] is highly similar to the 42- and 38-kD products encoded by HTLV-I and HTLV-II [6, 32]. That transactivating proteins play key roles in tumor induction and maintenance is strongly suggested by the observation that the 5'LTR and 3' region of the provirus are always conserved in BLV-induced tumors, even in cases where extended proviral deletions have occurred [12]. The lack of – or the very limited – expression of the *tat* gene product in BLV-induced tumors, even those propagated in vitro, argues in favor of BLV acting as an inducer of the neoplastic process and not as a maintenance determinant.

In this study we identified the protein product of the long open reading frame (LOR) gene, transcribed it in the SP6 system, and produced p34^x protein in reticulocyte lysates. The expressed native protein was used as an antigen to raise rabbit polyclonal antibodies. We further showed that most tumorous cattle and infected sheep

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harbor antibodies to p34^x. Combined with the transactivation effect observed by Rosen et al. [26] with the same gene construct, our data strongly suggest that p34^x is the *tat* gene product.

B. Results

Fetal lamb kidney (FLK)-BLV polyA⁺ RNA was used as a template for cDNA synthesis. The double-stranded cDNA, annealed with *Eco*RI linkers, was cloned in λ gt10. The library was screened with probes 1, 2, 3, and 4, as illustrated in Fig. 1. Accord-

ing to the known sequence of BLV provirus [22, 23, 27, 29], and in comparison with the HTLV-I and HTLV-II systems, it was inferred that the subgenomic mRNA coding for p34^x should contain sequences hybridizing to probes 1 and 2 but that it should be devoid of sequences complementary to probe 4. Probe 1 partly corresponds to the LOR encompassing the information for 308 amino acids. Probe 2 expands over the 3' end of the *pol* gene, the *env*, and the 5' part of the X region. Among the clones satisfying the above requirements, one (BL-1) was selected, subcloned in pBR322, amplified, and sequenced by the dideoxynucleotide

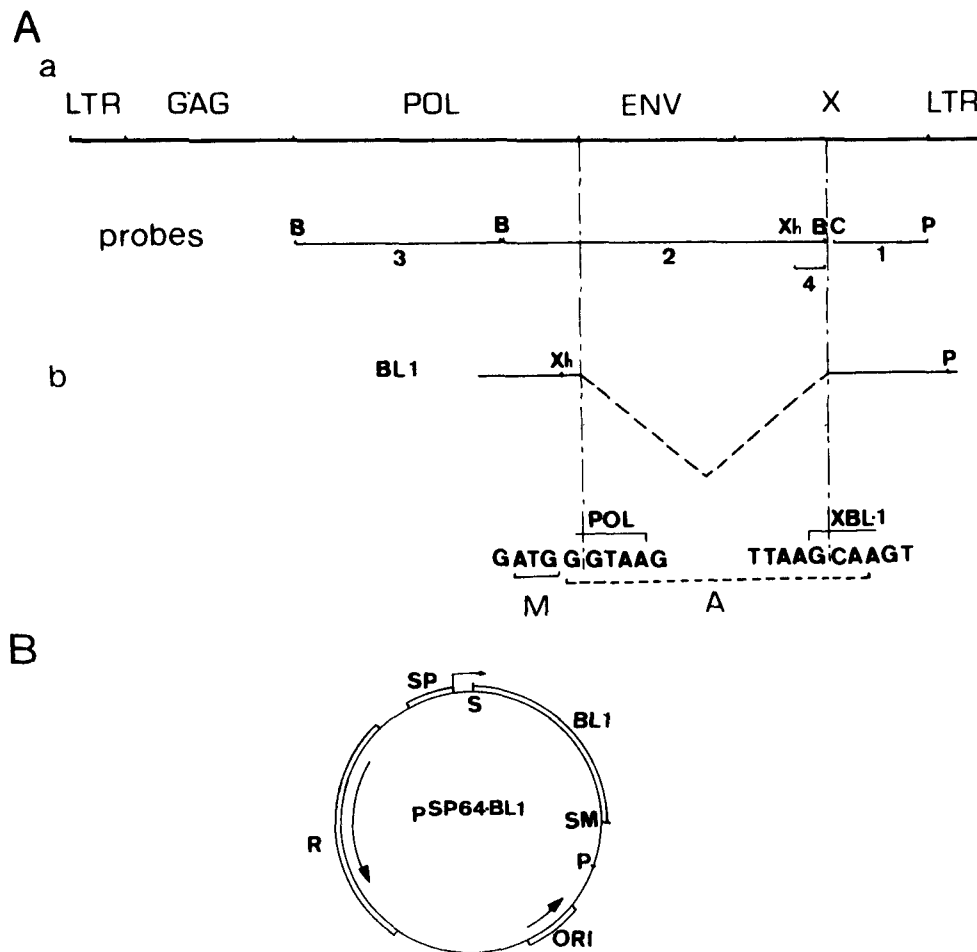


Fig. 1 A, B. Characterization of the BL1-cDNA and construction of the expression plasmid pSP64-BL1.

A a: Localization of the BLV probes on the BLV provirus. *B*, *Bam*HI; *C*, *Cla*I; *P*, *Pvu*II; *Xh*, *Xho*I. These probes were identified by DNA sequencing (not shown) and have been used previously [14]. **b:** Schematic representation of the BL1-cDNA. The splicing site between the methionine initiation codon (at positions 4868–4870) [28, 29] and the entire BL-1 LOR (beginning at posi-

tion 7246) generates an alanine (*A*) codon [30]. The two restriction endonucleases *Xho*I (*Xh*) and *Pvu*II (*P*) were used to subclone the coding region of BL-1 into the pSP64 plasmid.

B Construction of plasmid pSP64-BL-1. The *Xho*I-*Pvu*II fragment of BL-1 was subcloned into the *Sal*I (*S*)-*Sma*I (*SM*) sites of the pSP64 plasmid [17], according to Maniatis et al. [15]. *SP*, SP6 promoter; *ORI*, replication origin; *R*, ampicillin resistance gene; *P*, *Pvu*II

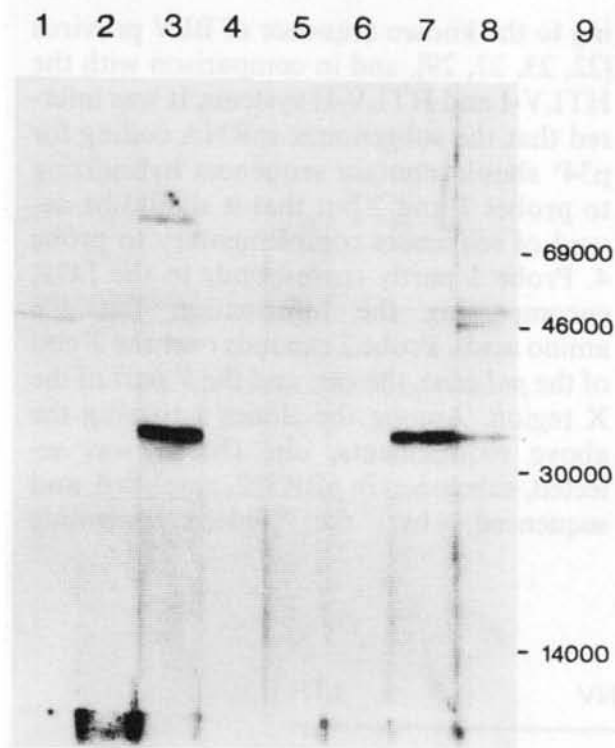


Fig. 2. Expression of p34^x. The techniques used were essentially those described by Butler and Chamberlin [1] and Renart and Sandoval [21]. Briefly, 5 µg of pSP64-BL1 DNA was digested to completion with *Pvu*II and transcribed in vitro by the SP6 RNA polymerase (Amersham) at 37 °C for 2 h. RNA was capped by means of a cap analogue (m⁷ G ppp G, Pharmacia) as described by the Amersham manufacturer. One microgram of the capped RNA was translated at 37 °C for 1 h in 20 µl of rabbit reticulocyte lysate supplemented with unlabeled amino acids [19]. The translation products were analyzed according to Renart and Sandoval [21]. The blotted p34^x protein was revealed by 50 µl of sera to be tested and 5 × 10⁴ cpm of ¹²⁵I-labeled protein A (specific activity: 3 × 10⁷ cpm/µg). Lane 1, normal rabbit serum; lane 2, rabbit anti-p24 polyclonal serum; lane 3, rabbit serum raised [7] against synthetic peptide RFPRDTSEPPLS of the p34^x protein [23]; lane 4, normal bovine serum; lanes 5 and 6, persistent lymphocytosis sera of cows 285 and 928 respectively; lanes 7 and 8, bovine tumor cases 15 and 82; lane 9, molecular weight markers

method. It was shown to contain 2353 base pairs consisting of 993 bp from the 3' end of the *pol* gene ending at the splice-donor sequence GATGG/GTAAG and of 1360 bp from the X region including 924 bp representing the LOR (or putative *tat* gene) and, starting at CAAGT, a fragment immediately following the splice-acceptor sequence

TCTTTTAAG (Fig. 1). The BL-1 clone thus derived from a spliced mRNA whose AUG is very close to the end of the *pol* message, 44 nucleotides downstream from the putative AUG of *env* mRNA.

The BLV restriction fragment (*Xho*I-*Pvu*II) containing the coding region of BL-1 was subcloned into the pSP64 plasmid. The recombinant plasmid was used to transform the HB101 strain of *Escherichia coli*. RNA from this clone was synthesized in vitro by the SP6 RNA polymerase and translated into a 34000-dalton protein (p34^x) in rabbit reticulocyte lysates.

Figure 2 illustrates Western blot experiments performed with reticulocyte lysates expressing p34^x. A 34000-dalton product is specifically recognized by a serum raised against a synthetic peptide whose sequence has been deduced from the putative COOH-terminus of the X LOR protein (lane 3). No p34^x was revealed by either normal (lane 1) or anti-BLV p24 (lane 2) rabbit serum. p34^x is also recognized by various sera from tumor-bearing BLV-infected cattle (lanes 7 and 8), although no p34^x was revealed by sera from animals with persistent lymphocytosis (lanes 5 and 6).

The data encountered in a limited epidemiological survey for anti-p34^x antibodies are summarized in Table 1. Most (22 of 24) bovine sera from tumor-bearing animals harbored anti-p34^x antibody, detectable by Western blotting. Many sera (6 of 8) from cattle with persistent lymphocytosis remained negative. Almost all BLV-infected sheep (23 of 25), whether in tumor phase or simply infected, without hematological dis-

Table 1. Response of BLV-infected animals to p34^x

Species	Stage of the disease	Western blot analysis	
		-	+
Cattle	Persistent lymphocytosis	6	2
	Tumorous case	2	2
Sheep	Antibody carrier to BLV gp51	0	
	Tumorous case	2	

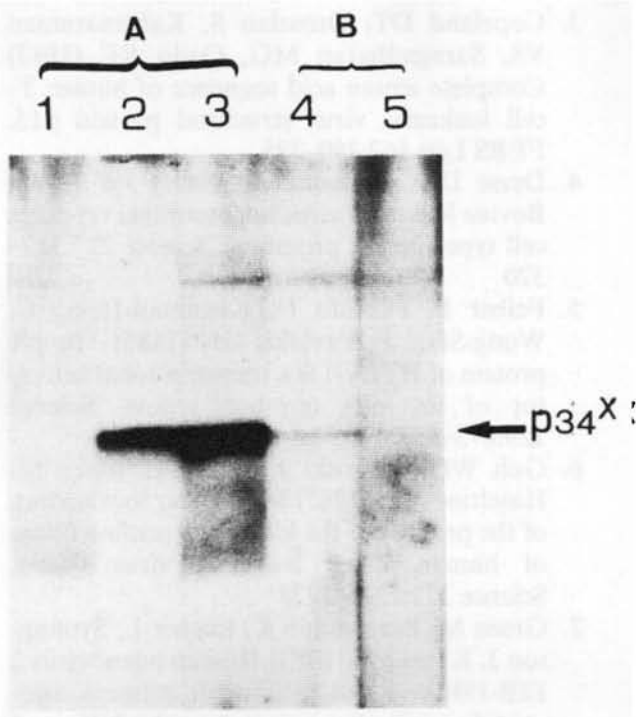


Fig. 3. Comparison of in vivo and in vitro synthesized p34^x. p34^x was expressed as described in the legend to Fig. 2. Subcellular localization of p34^x has been described previously [6]. *A*, In vitro synthesized p34^x. Lane 1, normal rabbit serum; lane 2, rabbit serum raised against synthetic peptide RFPRDTSEPPLS of the p34^x; lane 3, rabbit serum raised against p34^x made in reticulocyte lysates. *B*, FLK nuclei lysate. Lane 4, rabbit serum raised against synthetic peptide RFPRDTSEPPLS of the p34^x; lane 5, rabbit serum raised against p34^x

orders, reacted positively with p34^x. The latter result possibly reflects the high susceptibility of sheep to BLV infection and the high level of BLV replication in lymphoid and epithelioid sheep cells. Bovine cells are less susceptible to BLV replication, a fact reflected by the persistent, rather low antibody titer found in BLV-infected cattle. In general, anti-BLV antibody titers increase to high values (10⁵) only in the tumor stage of the disease. We are thus inclined to think that detection of anti-p34^x antibody in BLV-infected non-tumorous cattle might require even more sensitive techniques.

Reticulocyte lysates programmed by BL-1 RNA were injected (three successive injections of 40 µl lysates at 2-week intervals) into a rabbit and elicited an excellent anti-p34^x response. This rabbit polyclonal antibody (Fig. 3, lane 3) and the antisynthetic peptide rabbit serum (Fig. 3, lane 2) revealed

the presence of p34^x in SP64-BL-1 RNA-programmed reticulocyte lysates. The same antisera also recognized a p34^x product in a nuclear extract of BLV-infected FLK cells (FLK-BLV) (Fig. 3, lanes 4 and 5). The straightforward interpretation of these data is that the proteins made in vitro or in vivo are very similar, thus ruling out significant posttranslational modifications.

A plasmid expressing the BL-1 information was used in co-transfection experiments with a plasmid containing the chloramphenicol acetyl transferase (CAT) gene under the control of the BLV LTR. The spectacular increase of CAT expression demonstrates that p34^x is a powerful transactivator of BLV LTR in the cell system used [26].

It is an established fact that the level of transactivation and, hence, viral gene expression depend upon the cell line examined, thus suggesting that cell proteins are mandatory and play a role in proviral transcription [4, 5, 24–26, 31–34]. Through such interactions, it is commonly inferred that infection by HTLV-I, -II, or BLV leads not only to activation of viral genes but also to modification of expression of normal cellular genes, and this can be a first step toward malignant transformation. In the HTLV-I and -II systems, genes such as IL-2, the IL-2 receptor, and genes for class-II proteins of the major histocompatibility complex are target candidates for *tat* 1 [8] and *tat* 2. The functional similarity between the BLV and HTLV transactivator products suggests that expression of the *tat*_{BLV} product (p34^x) may induce expression of cellular genes involved in target cell proliferation. Overexpression of a few genes, however, does not explain the entire transformation process, because of the long latency period and monoclonality of the tumor. We are thus led to speculate that BLV-induced leukemogenesis is a multistep mechanism initiated by *tat*.

We propose two explanations for the fact that p34^x expression is the first event in a cascade leading to cell transformation and leukemia or lymphosarcoma. Moreover, the very limited expression of p34^x in cultured tumor cells indicates that maintenance of transformation is independent of p34^x expression [11].

Explanation 1: Expression of p34^x leads with low frequency to expression of a cell

protein critical for cell proliferation, modulating positively its own expression and acting as a repressor of p34^x expression. A model of this kind of interaction is the λ phage system in the interplay between genes C_{II} and C_I for establishment of lysogeny.

Explanation 2: Expression of p34^x induces with low frequency chromosomal abnormalities that definitely stabilize the transformed state. There are numerous examples of chromosomal rearrangements in neoplasia, such as the translocation of the Philadelphia chromosome in CML [9], the various translocations affecting the *myc* oncogene in Burkitt's lymphomas [35], and the chromosome breaks *bcl 1* and *bcl 2* in B cell lymphomas [36, 37]. Taking as an example a situation recently described in yeast [16], we imagine that p34^x expression leads with low frequency to an imbalance of histone-class proteins, which in turn induces chromosome abnormalities.

Cultured BLV-induced tumor cells systematically show karyotypic aberrations (Yu G, in preparation). It remains to be seen whether they are present in vivo or induced during establishment of the culture.

Finally, it can also be hypothesized that explanations 1 and 2 can be combined, chromosomal abnormality being a facultative consequence of p34^x expression.

Leukemogenesis by BLV is a complicated network of interactions, among which p34^x plays the initial role. It contributes to bringing the target cell into the transformed state; from then on, the cell proliferates and expands as the tumor clone.

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References

- Butler E, Chamberlin M (1982) Bacteriophage SP6 – specific RNA polymerase. *J Biol Chem* 257:5772–5778
- Chen I, McLaughlin J, Gasson JC, Clark SC, Golde DW (1983) Molecular characterization of genome of a novel human T-cell leukaemia virus. *Nature* 305:502–505
- Copeland DT, Oroszlan S, Kalyanaraman VS, Sarngadharan MG, Gallo RC (1983) Complete amino acid sequence of human T-cell leukemia virus structural protein p15. *FEBS Lett* 162:390–395
- Derse D, Caradonna SJ, Casey JW (1985) Bovine leukemia virus long terminal repeat: a cell type-specific promoter. *Science* 227:317–320
- Felber B, Paskalis H, Kleinman-Ening C, Wong-Staal F, Pavlakis GN (1985) The pX protein of HTLV-I is a transcriptional activator of its long terminal repeat. *Science* 229:675–677
- Goh WC, Sodroski J, Rosen C, Essex M, Haseltine WA (1985) Subcellular localization of the product of the long open reading frame of human T-cell leukemia virus type 1. *Science* 227:1227–1228
- Green M, Brackmann K, Lucher L, Symington J, Kramer T (1983) Human adenovirus 2 E1B-19K and E1B-53K tumor antigens; anti-peptide antibodies targeted to the NH₂ and COOH termini. *J Virol* 48:604–615
- Greene WC, Leonard WJ, Wano Y, Svetlik PB, Peffer NJ, Sodroski JG, Rosen CA, Goh WC, Haseltine WA (1986) Trans-activator gene of HTLV-II induces IL-2 receptor and IL-2 cellular gene expression. *Science* 232:877–880
- Heisterkamp N, Stephenson JR, Groffen J, Hansen PF, de Klein A, Bartram CR, Grosveld G (1983) Localization of the c-abl oncogene adjacent to a translocation breakpoint in chronic myelocytic leukaemia. *Nature* 306:239–242
- Kalyanaraman VS, Sarngadharan MG, Robert-Guroff M, Miyoshi I, Blayney D, Golde D, Gallo RC (1982) A new subtype of human T-cell leukemia virus (HTLV-II) associated with a T-cell variant of hairy cell leukemia. *Science* 218:571–573
- Kettmann R, Cleuter Y, Grégoire D, Burny A (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
- Kettmann R, Deschamps J, Cleuter Y, Couez D, Burny A, Marbaix G (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
- Lee TH, Coligan JE, Sodroski JG, Haseltine WA, Salahuddin JZ, Wong-Staal F, Gallo RC, Essex M (1984) Antigens encoded by the 3' terminal region of human T-cell leukemia virus: evidence for a functional gene. *Science* 226:57–61

14. Mamoun RZ, Astier-Gin T, Kettmann R, Deschamps J, Rebeyrotte N, Guillemain BJ (1985) The pX region of the bovine leukemia virus is transcribed as a 2.1-kilobase mRNA. *J Virol* 54:625–629
15. Maniatis T, Fritsch E, Sambrook J (eds) (1982) *Molecular cloning*. Cold Spring Harbor Lab., Cold Spring Harbor, N.Y.
16. Meeks-Wagner D, Hartwell LH (1986) Normal stoichiometry of histone dimer sets is necessary for high fidelity of mitotic chromosome transmission. *Cell* 44:43–52
17. Melton D, Krieg P, Rebagliati M, Maniatis T, Zinn K, Green M (1984) Efficient in vitro synthesis of biologically active RNA and RNA hybridization probes from plasmids containing a bacteriophage SP6 promoter. *Nucl Acid Res* 12:7035–7056
18. Oroszlan S, Sarngadharan MG, Copeland TD, Kalyanaraman VS, Gildea RV, Gallo RC (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
19. Pelham HRB, Jackson RJ (1976) An efficient mRNA-dependent translation system for reticulocyte lysates. *Eur J Biochem* 67:247–256
20. Poiesz BJ, Ruscetti FW, Reitz MS, Kalyanaraman VS, Gallo RC (1981) Isolation of a new type-C retrovirus (HTLV) in primary uncultured cells of a patient with Sezary T-cell leukaemia. *Nature* 294:268–271
21. Renart J, Sandoval IV (1984) Western blots. *Methods Enzymol* 104:455–460
22. Rice NR, Stephens RM, Burny A, Gildea RV (1985) The *gag* and *pol* genes of bovine leukemia virus: nucleotide sequence and analysis. *Virology* 142:357–377
23. Rice NR, Stephens RM, Couez D, Deschamps J, Kettmann R, Burny A, Gildea RV (1984) The nucleotide sequence of the *env* and post *env* region of bovine leukemia virus. *Virology* 138:82–93
24. Rosen CA, Sodroski JG, Kettmann R, Burny A, Haseltine WA (1985) Transactivation of the bovine leukemia virus long terminal repeat in BLV-infected cells. *Science* 227:320–322
25. Rosen CA, Sodroski JG, Kettmann R, Haseltine WA (1986) Activation of enhancer sequences in type-II human T-cell leukemia virus and bovine leukemia virus long terminal repeats by virus-associated trans-acting regulatory factors. *J Virol* 57:738–744
26. Rosen CA, Sodroski JG, Willems L, Kettmann R, Campbell K, Zaya R, Burny A, Haseltine WA (1986) The 3' region of bovine leukemia virus genome encodes a *trans*-activator protein. *EMBO J* 5:2585–2589
27. Sagata N, Yasunaga T, Ohishi K, Tsuzuku-Kawamura J, Onuma M, Ikawa Y (1984) Comparison of the entire genomes of bovine leukemia virus and human T-cell leukemia virus and characterization of their unidentified open reading frames. *EMBO J* 3:3231–3237
28. Sagata N, Tsuzuku-Kawamura J, Nagaioshi-Aia M, Shimizu F, Imagawa KI, Ikawa Y (1985) Identification and some biochemical properties of the major X_{BL} gene product of bovine leukemia virus. *Proc Natl Acad Sci USA* 82:7879–7882
29. Sagata N, Yasunaga T, Tsuzuku-Kawamura J, Ohishi K, Ogawa Y, Ikawa Y (1985) Complete nucleotide sequence of the genome of bovine leukemia virus: its evolutionary relationship to other retroviruses. *Proc Natl Acad Sci USA* 82:677–681
30. Seiki M, Hikokoshi A, Taniguchi T, Yoshida M (1985) Expression of the px gene of HTLV-I. General splicing mechanism in the HTLV family. *Science* 228:1532–1534
31. Slamon DJ, Shimotohno K, Cline MJ, Golde DW, Chen ISY (1984) Identification of the putative transforming protein of the human T-cell leukemia viruses HTLV-I and HTLV-II. *Science* 226:61–65
32. Slamon DJ, Press MF, Souza LM, Murdock DC, Cline MJ, Golde DW, Gasson JC, Chen ISY (1985) Studies of the putative transforming protein of the type-I human T-cell leukemia virus. *Science* 228:1247–1430
33. Sodroski J, Rosen C, Goh WC, Haseltine WA (1985) A transcriptional activator protein encoded by the X-LOR region of the human T-cell leukemia virus. *Science* 228:1430–1432
34. Sodroski JG, Rosen CA, Haseltine WA (1984) Transacting transcriptional activation of the long terminal repeat of human T-lymphotropic viruses in infected cells. *Science* 225:381–385
35. Taub R, Kirsch I, Morton C, Lenoir G, Swan D, Tronick S, Aaronson S, Leder P (1982) Translocation of the c-myc gene into the immunoglobulin heavy chain locus of human Burkitt lymphoma and murine plasmacytoma cells. *Proc Natl Acad Sci USA* 79:7837–7841
36. Tsujimoto Y, Finger LR, Yunis J, Nowell PC, Croce C (1984) Cloning of the chromosome breakpoint of neoplastic B cells with the (14;18) chromosome translocation. *Science* 226:1097–1099
37. Tsujimoto Y, Yunis J, Onorato-Showe L, Erikson J, Nowell PC, Croce C (1984) Molecular cloning of the chromosomal breakpoint of B-cell lymphomas and leukemias with the t (11;14) chromosome translocation. *Science* 224:1403–1406