Journal of General Virology (1997), **78**, 1941–1948. Printed in Great Britain

Use of the bovine leukaemia virus LTR U3 promoter for expressing antisense antiviral RNAs and competitive inhibition of viral infection in cell culture

D. Shayakhmetov, D. Kovalenko, G. Yurov, A. Borisenko and T. Tikchonenko

Department of Genetic Engineering, Institute of Agricultural Biotechnology, 42 Timiryazevskaya St, Moscow 127550, Russia

Use of viral inducible promoters which can be activated by virus-specific transactivator proteins to drive expression of antisense (as)RNA genes appears to be an attractive approach to inhibit virus infections *in vivo*. To this end, we have constructed an asRNA gene expressed from the bovine leukaemia virus (BLV) U3 promoter that is complementary to the R-U5 region of the BLV genome. This is the region that is most susceptible to inhibition by asRNA. With plasmid pLU, which expresses the asRNA gene under the control of the BLV U3 promoter, 75% inhibition of virus replication was attained in CC81 cells (the molar ratio of pLU DNA over BLV proviral DNA in the transfection mixture was 5:1). Plasmid pLT, which contains only the BLV U3 promoter without any asRNA-coding region, also efficiently (up to 60%) inhibited virus replication when cotransfected with BLV proviral DNA at a ratio of 20:1. It was suggested that competition between functional and 'empty' viral promoters for the viral transactivator protein p38^{tax} could account for this inhibition. An immunoblotting assay showed that in the presence of nuclear extracts from CC81 cells exogenous BLV p38^{tax} specifically associates with its responsive sequence located in the BLV U3 promoter. Moreover, the additional expression of p38^{tax} in CC81 cells abolishes the inhibitory effect of the empty viral promoter. These observations suggest a new mechanism of BLV inhibition caused, most probably, by sequestering of the viral transactivator protein.

Introduction

Antisense (as)RNAs have in many cases proved to be efficient antiviral agents in cell cultures as well as in host organisms (for references see: Takayama *et al.*, 1989; Rhodes & James, 1990; Miroshnichenko *et al.*, 1990; Kozireva *et al.*, 1996; Baurin *et al.*, 1995). Usually, strong promoters are used to drive the expression of asRNA genes. These promoters provide a large molar excess of asRNA molecules over target RNAs, which is important for effective inhibition of virus replication (Cotten & Birnstiel, 1989; Wang & Dolnick, 1993). At the same time, in the absence of viral infection it seems reasonable to keep the expression of asRNA genes at the lowest possible level. This consideration is of special importance in experiments with transgenic animals where all cells of the organism contain the expressed transgene. Due to the limited tropism of virus infections, most host tissues and cells remain free of virus but still produce what is at best useless asRNA. This problem can be easily solved *in vitro* by using inducible promoters, but this approach does not work well in the case of transgenic animals. To express antiviral asRNA genes, it is much more desirable to use viral promoters that are activated by cognate viral transactivators. It has been shown previously that the bovine leukaemia virus (BLV) U3 promoter is activated by the virus-encoded protein p38^{tax}, most probably via formation of a complex with CREB2, a cellular protein of the CREB/ATF family (Katoh *et al.*, 1987; Willems *et al.*, 1992; Kiss-Toth *et al.*, 1993). By designing an asRNA construct of this type, we anticipated that the BLV-specific activator would launch and control transcription of the R-U5 asRNA gene, and that this transcription would be proportional to the level of virus replication and be limited to infected cells. Antiviral activities and expression patterns of asRNA genes driven by cognate viral promoters have never been examined.

In this paper, transcription of an R-U5-targeted asRNA gene and of a β-Gal reporter gene driven by BLV U3 promoters was characterized in CC81 cells. Evaluation of the antiviral

Author for correspondence: T. I. Tikchonenko.
Fax +7 095 977 0947. e-mail thomas@agrobio.msk.su

0001-4597

activity of the asRNA gene conclusively attests that efficient inhibition of virus replication can be achieved at rather low (2:1 to 5:1) molar excesses of cotransfected asRNA-coding DNA over BLV proviral DNA. Inhibition of BLV replication in CC81 cells under these conditions was over 75%. At the same time, it was observed that cotransfection of a plasmid containing the 'empty' BLV U3 cassette devoid of any asRNA genes along with BLV proviral DNA at high (up to 20:1) molar ratios also results in efficient inhibition of virus replication. Since the transcriptional activity of the BLV U3 promoter is highly dependent on the presence of the viral protein $p38^{tax}$, we concluded that cotransfected BLV promoter can trap this protein and decrease its intracellular concentration, thus inhibiting virus replication.

Since there has, so far, been no direct evidence that BLV $p38^{tax}$ physically interacts with its responsive sequences within the viral promoter, we examined whether such interaction occurs *in vitro*. Using an immunoblotting assay, we demonstrated that $p38^{tax}$, in the presence of nuclear extract from CC81 cells, does associate with its responsive element within the viral promoter in a manner similar to that of the $p40^{tax}$ protein of human T-lymphotropic virus I (HTLV-I) (Marriott *et al.*, 1989). Moreover, if the $p38^{tax}$-expressing plasmid was added to the transfection mixture, the inhibitory effect of the empty BLV promoter was totally abolished. Marked similarities in genomic structures and transcriptional mechanisms between BLV and lymphotropic human retroviruses like HTLV-I and HTLV-II (Sagata *et al.*, 1985; Orita *et al.*, 1993) allow use of BLV and the BLV–asRNA system as a model for developing new antiviral agents for therapy and prophylaxis of this important group of viral infections. Thus, a new approach to antiviral therapies based on competitive binding of transactivator proteins to enhancer sequences in viral promoters and exogenous DNAs is proposed.

Methods

■ **Design of recombinant plasmids.** All recombinant DNA manipulations (cloning, transfection, purification of plasmid DNA) were performed according to the protocols described by Sambrook *et al.* (1989). The following plasmids were used: pUC19 (Boehringer Mannheim); pGEM-7Z and pGEM-3Z (Promega); pBC-Bluescript (Stratagene); pB6490 with the BLV progenome, kindly provided by K. Sekikawa (National Institutes of Animal Health, Japan) and described in detail by Itohara *et al.* (1987); pAG60 with the *neo*-gene cassette, consisting of the herpes simplex virus type 1 (HSV-1) thymidine kinase (TK) promoter and termination-polyadenylation sequences and the *neo* gene (Colbere-Garapin *et al.*, 1981); pEQ176 with the β-Gal gene driven by the IE CMV promoter (gift from J. Overbaugh, University of Washington, Seattle, USA); and pSGtax, expressing $p38^{tax}$ under the control of the SV40 promoter, obtained from L. Willems, Gembloux University of Belgium, Brussels, Belgium and described in detail by Willems *et al.* (1987). All enzymes used in this study were purchased from Promega and BRL; radiolabelled [α-^{32}P]UTP and [α-^{32}P]CTP were from Amersham.

Plasmid pGL, carrying the β-Gal gene downstream of the BLV U3 promoter, was constructed using pEQ176 as a backbone. Its immediate early cytomegalovirus (IE CMV) promoter was replaced with the BLV U3 promoter using the flanking *Eco*RI and *Sac*I sites. To construct the pLT vector, carrying the BLV U3 promoter, we excised it from pB6490 and inserted it between the *Eco*RI and *Sac*I sites of pUC19. The signal for termination of transcription and polyadenylation was excised from pAG60 with *Sma*I and *Pvu*II and inserted into the plasmid DNA at the *Sma*I site. Finally, to construct pLU, the *Sac*I–*Bcl*I fragment of the BLV genome (185 bp) was cloned at the *Sma*I site of pLT in reverse orientation relative to the BLV U3 promoter.

■ **Cell lines, transfection, and plasmid DNA quality assays.** CC81 feline cells (Ferrer *et al.*, 1981) were grown at 37 °C and 5% CO_2 in DMEM supplemented with 10% foetal bovine serum (HyClone), 100 units/ml of penicillin and 100 μg/ml of streptomycin. The cells were transfected with plasmid DNA (purified by two cycles of centrifugation in a CsCl gradient) using the calcium phosphate co-precipitation technique (Graham & van der Eb, 1973). DNA concentration was estimated by measuring the absorbance at 260 nm.

When the antiviral effect of an asRNA gene is to be estimated, cotransfection of cells with a mixture of different plasmid DNAs requires choosing DNA samples that will not affect the transfection efficiency at different molar ratios. To this end, 1×10^5 CC81 cells were seeded in a 35 mm dish 1 day before transfection. The next day, 1 μg of plasmid expressing the β-Gal gene was cotransfected with 10 μg of the plasmid DNA being tested as a carrier. Forty-eight hours post-transfection the cells were fixed, covered with X-Gal-containing solution to detect transient β-Gal expression, and, on the next day, the bright-blue cells on each dish were counted. Only plasmid DNA samples differing in transfection efficiency assay by less than 10% of the average were used in subsequent experiments.

■ **Syncytia test.** The syncytia test was used to evaluate the inhibitory effect of asRNA on replication of BLV in CC81 cells (Borisenko *et al.*, 1992). CC81 cells ($1{\cdot}7 \times 10^5$) were seeded into a 35 mm dish 1 day before transfection. Twenty-four hours later, semiconfluent monolayers were cotransfected with mixtures of BLV proviral DNA from plasmid pB6490 (1 μg) and either carrier DNA (pGEM-3Z or pGEM-7Z) or pLT or pLU. The total amount of transfected DNA in each experiment was adjusted with pGEM-7Z to 11 μg per dish. Twenty-four hours post-transfection, cells from one dish were trypsinized, split in two aliquots and cultured for a further 5 days at 37 °C and 5% CO_2 in DMEM supplemented with 10% foetal bovine serum, 1% DMSO and 4 μg/ml polybrene. Monolayers were then fixed with 2·5% glutaraldehyde, stained with 0·1% Toluidine Blue in PBS, and the number of syncytia was counted.

The inhibitory effect of an asRNA gene was calculated as $(1-N_{\mathrm{pLU}}/N_{\mathrm{pLT}}) \times 100\%$, where N_{pLU} and N_{pLT} are numbers of syncytia formed in cells transfected with pLU and pLT, respectively, at the same molar excess of plasmid DNA over BLV proviral DNA. The inhibitory effect of the pLT vector was calculated as $(1-N_{\mathrm{pLT}}/N_{\mathrm{BLV}}) \times 100\%$, where N_{BLV} is the number of syncytia formed in cells transfected with BLV proviral DNA at the same molar excess of plasmid or carrier DNA over BLV proviral DNA.

■ **Isolation of total cellular RNAs.** Total RNAs were isolated by lysing cells in acidic 4 M guanidine isothiocyanate and extracting with a phenol–chloroform mixture as described by Ausubel *et al.* (1991). RNA preparations were precipitated twice with isopropanol, washed with 75% ethanol, and dissolved in autoclaved water treated with 0·1% diethylpyrocarbonate. Concentration and purity of RNA were estimated by measuring the absorbance ratio at 260/280 nm; this usually exceeded 1·8. On average, the yield was 100 μg of RNA from 1×10^7 cells.

■ ***In vitro* synthesis of ^{32}P-labelled RNA probes and RNase protection assay.** The ^{32}P-labelled RNA probes were obtained using the Riboprobe-II *in vitro* transcription system (Promega) according to the manufacturer's protocol. The *Sac*I–*Bcl*I fragment of the BLV genome (nt 365–551 according to Sagata *et al.*, 1985) was blunt-ended with T4 DNA polymerase and cloned into the *Sma*I site of the polylinker of pGEM-7Z. Subsequent *in vitro* transcription initiated from the SP6 promoter yielded an RNA probe complementary to the antisense transcript of pLU. Similarly, the 385 bp *Pvu*II–*Pvu*II fragment from the 3′ end of the *β*-Gal gene was cloned into pGEM-7Z at the *Sma*I site to yield, after transcription from the SP6 promoter, an RNA probe complementary to the *β*-Gal mRNA. To provide run-off transcripts, these plasmids were cut with *Eco*RI, purified, and incubated for 1 h at 40 °C, according to the manufacturer's protocol. After incubation, 5 units of RNase-free DNase (Promega) was added to the reaction mixture and incubated for 15 min. After a single treatment of the RNA solution with phenol–chloroform–isoamyl alcohol (25:24:1), RNA was precipitated with ethanol in the presence of carrier tRNA. Finally, the RNA probe was purified by elution from a 6% denaturing polyacrylamide gel as described by Ausubel *et al.* (1991). The average specific activity of the RNA probe was $1{\cdot}5 \times 10^8$ c.p.m./μg.

RNase protection assays were performed according to the Ambion instruction manual (RPA II) without any modifications. Total cell RNA (40 μg) was hybridized with RNA probe overnight at 45 °C for the pLU antisense probe and at 53 °C for the *β*-Gal probe in 30 μl of hybridization buffer containing 80% formamide, 40 mM PIPES pH 6·4, 400 mM NaCl and 1 mM EDTA. Digestion of ssRNA was performed for 1 h at 37 °C with 6 μg/ml RNase A and 12 U/ml of RNase T_1 (Sigma) in RNase buffer containing 300 mM NaCl, 5 mM EDTA and 10 mM Tris–HCl pH 7·5. After recovery of RNA by ethanol precipitation, samples were analysed by electrophoresis in 8% denaturing gels. Dried gels were exposed to X-Omat X-ray film (Kodak) at −70 °C overnight. Molecular size markers were [α-^{32}P]dATP-labelled *Hpa*II fragments of pGEM-3Z or *Hin*fI fragments of ϕX174 DNA end-labelled with polynucleotide kinase and [γ-^{32}P]ATP.

■ **RT–PCR analysis and Southern blotting.** To identify specific transcripts of the asRNA gene in CC81 cells infected with BLV, an RT–PCR method, described in detail by Kozireva *et al.* (1996), and Southern blotting were used. Briefly, 20 μg of total RNA isolated from CC81 cells cotransfected with an asRNA gene-containing plasmid and BLV proviral DNA was treated with 10 U of RNase-free DNase (Sigma). The RNAs were purified from the enzyme and 5 μg of each RNA was used in a standard reverse transcriptase reaction (RT System A3500, Promega; manufacturer's protocol) containing the specific forward primer [5′ GCTCTATCTCCGGTCCTCTG 3′, nt 355–375 (Sagata *et al.*, 1985)] to initiate synthesis of cDNA from asRNA molecules. Then, a 1/5 vol. RT reaction mixture was amplified in a PCR, which also included the reverse primer [5′ GTTTGCCCGGTCTCCCTGG 3′, nt 510–529 (Sagata *et al.*, 1985)]. The amplification was carried out as described in Innis *et al.* (1990). The length of the expected specific fragment was 174 bp. Total RNAs (5 μg) treated with RNase-free DNase only (without RT treatment) were used as a negative control in the PCR assay. PCR was carried out with AmpliTaq DNA polymerase (Perkin-Elmer) for 30 cycles (94 °C for 60 s, 58 °C for 60 s and 72 °C for 20 s), followed by electrophoresis in a 2% agarose gel and transfer to a positively charged nylon membrane (Hybond-N+, Amersham). The oligonucleotide 5′ GGCGCCGAAGG-AGAGAGCGCGGG 3′ [nt 479–501 (Sagata *et al.*, 1985)] was end-labelled with polynucleotide kinase and [γ-^{32}P]ATP and used as a probe to detect the asRNA-specific PCR product. Hybridization was carried out using Rapid-hyb buffer (Amersham) and an oligonucleotide probe according to the manufacturer's protocol without any modifications, and was followed by autoradiography.

■ **Indirect DNA binding assay.** The 24 nt oligonucleotide 5′ GCTGGTGACGGCAGCTGGTGGCTA 3′, corresponding to the CRE/ATF responsive element core sequence within the BLV U3 promoter [nt −113 to −137 (Sagata *et al.*, 1985)] and its antisense counterpart, as well as an oligonucleotide with randomized sequence (5′ CTGATGAGTCCGTGAGGACGA 3′) and its antisense counterpart, were synthesized by a standard technique. For a subsequent immunoblotting assay, three T residues were added to the 5′ end of each oligonucleotide. Both pairs of oligonucleotides were annealed in 6 mM Tris–HCl pH 7·5, 0·05 M NaCl, 8·7 mM $MgCl_2$ by incubating for 5 min at 97 °C and slowly cooling to room temperature. After ethanol precipitation, the termini of the annealed double-stranded oligonucleotides were in-filled with biotinylated-dATP (Sigma) using Klenow DNA polymerase. Nuclear and cytoplasmic extracts were prepared as described by Hirai *et al.* (1992). The biotinylated oligonucleotides (1 μg) were mixed in 10 mM Tris-HCl pH 7·5, 40 mM NaCl, 1 mM dithiothreitol, 1 mM EDTA, 0·25% Triton X-100 with cell lysates (8 μg of protein) containing poly(dI·dC) (5 μg) in the presence or absence of p38^{tax} protein (50 ng). The mixture was incubated for 30 min on ice. Then an excess of streptavidin MagneSphere particles (Promega) was added and incubated for 30 min at room temperature with gentle agitation. The MagneSphere particles were collected with a magnet and washed twice with 5 vols (0·5 ml) of buffer. The trapped proteins were subjected to SDS–PAGE (15% separating gel), followed by electrophoretic transfer to an Immobilon-P transfer membrane (Millipore) and immunoblotting. The antibodies used were mouse anti-p38^{tax} Igs. The bands were detected using anti-mouse IgG–horseradish peroxidase conjugate and 3,3′-diaminobenzidine. The p38^{tax} protein and antibodies against it were kindly provided by L. Willems.

Results

Enhanced transcription of the BLV U3-driven asRNA gene in CC81 cells

The transcriptional activity of the BLV U3 promoter in the absence of the viral transactivator protein p38^{tax}, termed basal transcriptional activity, is very low (Powers & Radke, 1992). In order to characterize basal and enhanced transcription from the BLV U3 promoter and to check the expression of the constructed asRNA gene, CC81 cells were transfected with pLU (containing the R-U5 asRNA gene downstream of the BLV U3 promoter) or with a mixture of pLU and pSGtax, a p38^{tax}-expressing plasmid (Willems *et al.*, 1987). As a control we used pSGtax(−), a pSGtax derivative from which the SV40 promoter has been removed. The RNase protection assay revealed a rather low basal level of transcriptional activity of the BLV U3 promoter in CC81 cells (about 50 asRNA molecules per cell, judging by the quantitative RNase protection assay; data not shown). By contrast, expression of the BLV U3 promoter-driven asRNA gene was significantly higher (15-fold) in cells transfected with a mixture of pLU + pSGtax (Fig. 1). This enhanced asRNA gene expression confirmed that the BLV U3 promoter is strongly transactivated by p38^{tax} in CC81 cells. This result is in good agreement with data reported from several studies on transactivation by p38^{tax} as well as

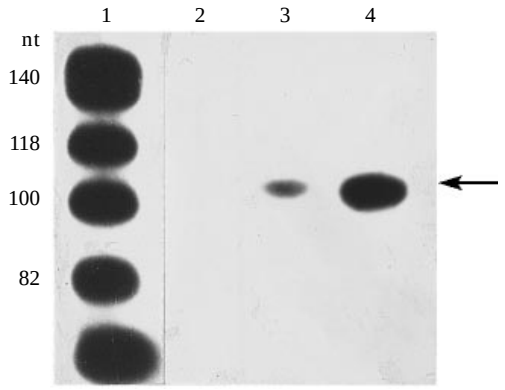

Fig. 1. Activation of the BLV U3 promoter-driven asRNA gene by the p38^{tax} transactivator. CC81 cells were transfected with pGEM-3Z alone (negative control, lane 2), pLU + pSGtax(−) (lane 3) or pLU + pSGtax (lane 4). Forty-eight hours post-transfection cells were harvested and total cellular RNAs were isolated and assayed to determine asRNA gene expression. Lane 1, molecular size marker. The specific asRNA transcript is indicated by the arrow.

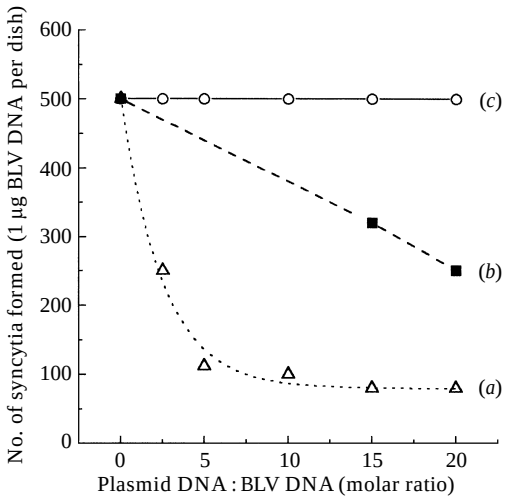

Fig. 2. Number of syncytia formed by CC81 cells cotransfected with different plasmids and BLV proviral DNA. Curve (*a*), cells cotransfected with BLV DNA + pLU (the asRNA gene driven by the BLV U3 promoter); curve (*b*), cells cotransfected with BLV DNA + pLT (BLV U3 promoter); curve (*c*), cells cotransfected with BLV DNA + pGEM-3Z as a control.

BLV U3 promoter activity in other cell lines (Derse, 1988; Katoh *et al.*, 1989).

Dose-dependent inhibition of BLV replication by the asRNA gene driven by the viral promoter

To assess inhibition of BLV replication by the BLV U3 promoter-driven asRNA gene, a syncytia formation assay as well as an RNase protection assay were used. CC81 cells were cotransfected with equal amounts of BLV proviral DNA and increasing amounts of other plasmids (Fig. 2): pGEM-3Z (as a control DNA, curve *c*); pLT, carrying just the BLV U3 promoter (curve *b*) or pLU, an asRNA gene-expressing plasmid (curve *a*). In all these experiments the total amount of cotransfected DNA was adjusted with carrier pGEM-7Z DNA to 11 μg per 35 mm dish. When pGEM-3Z or pGEM-7Z were transfected alone, no syncytia were formed (data not shown). The molar ratio of BLV proviral DNA to pLT or pLU DNAs varied from 1:1 to 1:20. Fig. 2 shows that transfection of 1 μg of BLV proviral DNA per dish of CC81 cells results, on average, in formation of 500 syncytia. When the CC81 cells were transfected with a mixture of BLV DNA + pLU DNA expressing the asRNA gene, the number of syncytia formed was significantly reduced. Thus, even at low (about 2:1) molar ratios of asRNA-coding DNA to BLV DNA the antiviral effect of asRNA was evident and the number of syncytia was almost 50% lower compared with the control. Analysis of genomic viral RNA in cells transfected with the mixture of asRNA-encoding plasmid and BLV proviral DNA revealed (Fig. 3) that the amount of viral RNA was reduced markedly if a 5:1 or 10:1 molar excess of the asRNA-encoding plasmid was used. However, the correlation between the amount of pLU DNA in the transfection mixture and the extent of BLV inhibition was not simple. At a 1:5 ratio of proviral to asRNA-encoding DNA, the inhibitory effect reached 75% (about 100 syncytia per dish); this value did not change significantly when higher molar excesses of the asRNA-encoding DNA were used.

The data obtained in these experiments revealed a rather interesting feature of pLT, which contains just the BLV U3 promoter without any coding genes. When pLT was co-transfected in high molar excesses over BLV proviral DNA (Fig. 2, curve *b*), this plasmid inhibited BLV replication only slightly less efficiently than the asRNA-coding construct. When CC81 cells were transfected with the mixture of BLV DNA and pLT DNA at a molar ratio of 1:20, syncytia formation was reduced by 60%. In contrast to curve (*a*) in Fig. 2, curve (*b*) is practically linear, attesting the existence of another molecular mechanism, distinguishable from that of asRNA, and responsible for the observed inhibition of virus reproduction.

Analysis of BLV U3 promoter-driven gene transcription in CC81 cells infected with BLV

To investigate in detail the transcriptional activity of the BLV U3 promoter in CC81 cells infected with BLV, we tried to detect the asRNA molecules by using a quantitative RNase protection assay. However we failed, probably due to the low level of free asRNA, most of which is probably bound to the target BLV RNA. Nevertheless, analysis of total RNA isolated from cells cotransfected with the asRNA-encoding plasmid and BLV proviral DNA at a ratio of 2:1, using an RT–PCR assay followed by Southern blotting, clearly demonstrated the presence of asRNA molecules in transfected cells (Fig. 4).

For quantitative evaluation of BLV U3 transcriptional activity in infected cells we again used the RNase protection

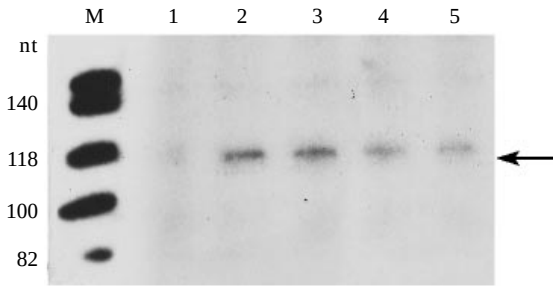

Fig. 3

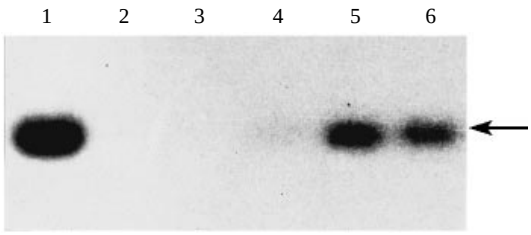

Fig. 4

Fig. 3. Decreased levels of viral genomic RNA in cells cotransfected with plasmid pLU and BLV proviral DNA. Lane 1, total RNA from CC81 cells; lane 2, RNA from cells cotransfected with BLV proviral DNA and pGEM-3Z at a molar ratio of 1:10; lanes 3, 4 and 5, RNA from cells cotransfected with BLV progenomic DNA and plasmid pLU at molar ratios of 1:1, 1:5 and 1:10, respectively. Lane 1, molecular size marker. The specific BLV genomic RNA transcript is indicated by the arrow.

Fig. 4. Expression of the asRNA gene driven by the BLV U3 promoter in CC81 cells transfected with the BLV proviral genome. Duplicate RNA samples from CC81 cells were treated with RNase-free DNase, and used for RT–PCR followed by Southern blotting (lanes 5 and 6). The same DNase-treated RNA samples were directly amplified in control sets (lanes 3 and 4). *E. coli* tRNA was used as a negative control in the RT–PCR assay (lane 2), and 10 ng of the 174 bp PCR product from the same part of the BLV genome was used as a positive control for Southern blotting (lane 1).

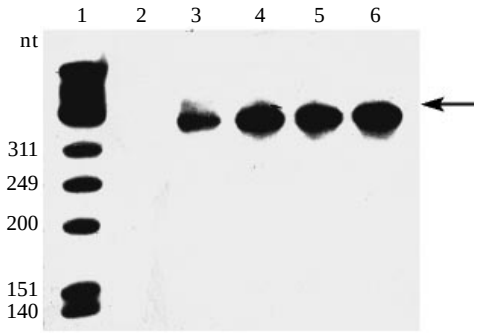

Fig. 5. Transcription of the β-Gal gene driven by the BLV U3 promoter in CC81 cells cotransfected with pGL and BLV proviral DNA. Lane 1, molecular size marker; lane 2, RNA from cells transfected with BLV proviral DNA; lanes 3, 4, 5 and 6, RNAs from cells cotransfected with a mixture of pGL and BLV DNA at molar ratios of 1:1, 3:1, 6:1 and 10:1, respectively. The specific β-Gal transcript is indicated by the arrow.

assay and plasmid pGL, which contains the β-Gal reporter gene downstream of the BLV U3 promoter. According to computer analysis of the β-Gal gene and the BLV genome, their transcripts cannot form duplexes and so affect the amount of detectable β-Gal gene transcripts. CC81 cells were cotransfected with a mixture of 10 μg of BLV proviral DNA and either 90 μg of pGEM-3Z, a control DNA, or increasing amounts of pGL DNA (from 10 to 90 μg per flask; molar ratios of pGL DNA to BLV DNA 1:1, 3:1, 6:1 and 10:1). The amount of total DNA transfected in each experiment was adjusted to 100 μg with pGEM-3Z. Forty-eight hours post-transfection total cellular RNA was extracted, purified, and the level of β-Gal mRNA was measured. Fig. 5 demonstrates that an increase in the molar ratio of pGL over BLV proviral DNA from 1:1 to 3:1 results in increased transcription of the reporter gene driven by the BLV U3 promoter. At the same time, when higher molar ratios of pGL over BLV DNA (6:1 and 10:1) were used, the levels of β-Gal mRNA did not increase significantly. To investigate whether the observed saturation is due to the inability of CC81 cells to take up large amounts of DNA, we cotransfected CC81 cells (8×10^6 per 75 cm^2 flask) with increasing amounts (from 10 to 150 μg) of pGL without BLV DNA. As evidenced by the RNase protection analysis of total cellular RNAs, there is a strictly linear dependence between the amount of transfected plasmid DNA and the level of β-Gal mRNA transcripts, at least in the range of DNA concentrations tested (data not shown). Thus, we concluded that BLV U3 promoter transcriptional activity in transfected cells is activated by transcriptional factors of replicating virus and, at the same time, is highly dependent on the molar excess of unoccupied ('empty') BLV U3 promoters in infected cells compared with viral promoters. As the amount of transactivator protein in cells is limited we suggest that competition for transcription factors plays a major role in BLV inhibition by the BLV U3 promoter.

Cotransfection of p38^{tax}-expressing plasmid with BLV proviral DNA abolishes the inhibitory activity of the BLV U3 promoter

If the suggested competition between BLV U3 promoters (in pLT) and BLV promoter in proviral DNA is the cause of inhibition of BLV replication in cotransfected cells (Fig. 2, curve *b*), then it should be abolished by overexpression of the limiting transcriptional factor. We therefore cotransfected CC81 cells with a mixture of pLT DNA + BLV proviral DNA at a ratio of 20:1 and increasing amounts (from 1 to 3 μg per dish) of pSGtax, expressing BLV p38^{tax} under the control of the SV40 early gene promoter. The data obtained clearly show that addition of the p38^{tax}-expressing plasmid to the trans-

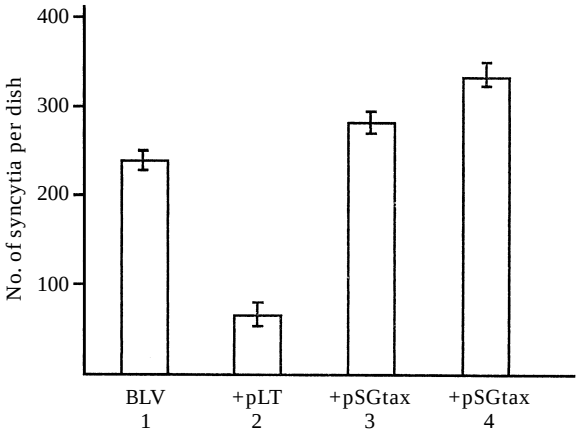

Fig. 6. Number of syncytia formed by CC81 cells cotransfected with a mixture of pLT + BLV proviral DNA and different amounts of the p38^{tax}-expressing plasmid pSGtax. Column 1, cells cotransfected with BLV proviral DNA + pSGtax (−); column 2, cells cotransfected with BLV DNA + pLT with LTR U3 promoter at a ratio of 1:20; column 3, as column 2, but plus 1 μg pSGtax per dish; column 4, as column 2, but plus 3 μg pSGtax per dish.

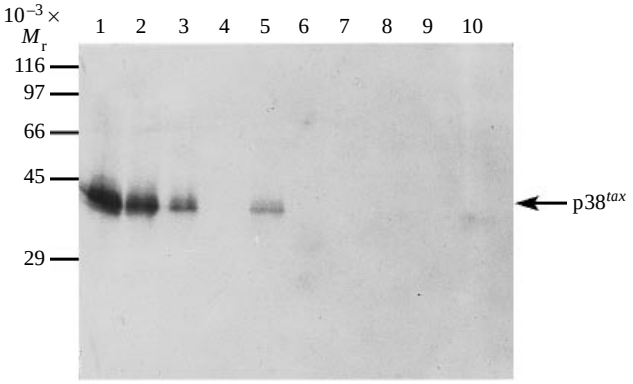

Fig. 7. Complex formation between exogenous p38^{tax} and the 24 bp enhancer DNA sequence in nuclear and cytoplasmic extracts of CC81 cells. Biotinylated wild-type oligonucleotides were incubated in cytoplasmic (lanes 4 and 6) or nuclear (lanes 5 and 7) extracts of CC81 cells in the absence (lanes 6 and 7) or presence (lanes 4 and 5) of exogenous p38^{tax}. The same oligonucleotides were incubated with p38^{tax} in the absence of cytoplasmic or nuclear extracts (lane 8). Biotinylated oligonucleotides of randomized sequence were incubated with p38^{tax} in the presence of cytoplasmic (lane 9) or nuclear (lane 10) extracts of CC81 cells. Lane 3, wild-type oligonucleotides were incubated with nuclear extracts of CC81 cells transfected with pSGtax. The isolated DNA–protein complexes were analysed by immuno-blotting using anti-p38^{tax} monoclonal antibodies. Lanes 1 and 2 contain purified p38^{tax} (50 and 25 ng, respectively). M_r markers are indicated.

fection mixture strongly activates BLV replication and totally abolishes the antiviral effect of pLT (Fig. 6). The presence of p38^{tax} protein in CC81 cells transfected with pSGtax can be easily demonstrated by immunoblotting assay (see Fig. 7, lane 3). Thus, we conclude that under the above conditions the viral transcriptional factor p38^{tax} is the limiting factor suppressing efficient transcription from the BLV U3 promoter, and that competition for this transactivator appears to be the mechanism by which BLV replication is inhibited by pLT.

Indirect binding of p38^{tax} to the CRE/ATF responsive element in the BLV U3 promoter

As competition for p38^{tax} appeared to be the mechanism for inhibition of BLV replication by pLT, we performed a further experiment to show more rigorously that p38^{tax} does associate with its responsive element located in this promoter. The BLV U3 promoter harbours, in three separate parts, short stretches matching the consensus sequence for the cAMP-responsive element (CRE) and for binding sites for a cellular transcriptional activator, ATF. A mutation in this sequence totally abolished the responsiveness of the promoter (Katoh *et al.*, 1989). We have chosen one such segment (nt −113 to −137; Sagata *et al.*, 1985) to examine whether p38^{tax} associates with this sequence. To demonstrate interaction of this protein with the CRE/ATF enhancer element in CC81 cells, we used an indirect DNA-binding assay, applied previously to demonstrate binding of the HTLV-I p40^{tax} protein to its responsive sequence and described in detail by Marriott *et al.* (1989). Double-stranded oligonucleotides containing the −113 to −137 sequence of the viral promoter were labelled with biotinylated-dATP and incubated with nuclear or cytoplasmic extract of CC81 cells and purified p38^{tax}. The DNA–protein complexes formed were isolated with streptavidin MagneSphere particles as described in Methods, and were examined by immunoblotting using an antibody against p38^{tax}. When wild-type oligonucleotides were used, p38^{tax} protein was detected in the DNA–protein complexes, which were formed only in the presence of nuclear rather than cytoplasmic extract (Fig. 7, lanes 4 and 5). Thus, nuclear factors appear to be essential for the formation of the complex between p38^{tax} and the CRE/ATF sequence in the BLV U3 region. Endogenous p38^{tax} complexed with the DNA probe was not detected in CC81 cell extracts (lanes 6 and 7). Also, p38^{tax} could not by itself associate with the wild-type oligonucleotides (lane 8). To control for non-specific DNA binding by p38^{tax}, we used a biotinylated double-stranded oligonucleotide of randomized sequence, to which only a trace amount of p38^{tax} could bind and only in the presence of nuclear extract of CC81 cells (lanes 9 and 10).

Discussion

In the present study, it has been demonstrated for the first time that transactivator-controlled viral promoters, like BLV U3, can be successfully used to drive the expression of an asRNA gene targeted at the genomic RNA of the same virus. Recently, use of the BLV LTR promoter to transcribe the gene coding for diphtheria toxin A chain for selective elimination of cells transformed with BLV has been reported (Kakidani *et al.*, 1993). Such asRNA genes might overcome one of the

unwanted consequences of asRNA-based informational immunity intended to be used in transgenic multicellular organisms (Coleman *et al.*, 1985). Usually, in transgenic animals resistant to a certain viral infection, all cells express the asRNA gene constitutively. However, most of these cells will never be hit by the virus due to the well-known tissue- and cell-tropisms of viral infections. The ideal asRNA constructs must provide, therefore, expression of antiviral asRNA genes only in those cells of the organism which can be infected by the virus, and only when they are infected. Our present study demonstrates that these criteria can be met, at least partially. Expression of an anti-BLV asRNA gene driven by the cognate viral promoter was practically undetectable in CC81 cells in the absence of the viral transactivator protein $p38^{tax}$. However, cotransfection of CC81 cells with plasmids containing the asRNA gene and a $p38^{tax}$-expressing plasmid (pSGtax) caused a more than 15-fold increase in expression of the asRNA gene (Fig. 1). Similar data were obtained when plasmid containing the β-Gal gene under the control of the BLV U3 promoter was cotransfected with BLV proviral DNA into CC81 cells (Fig. 5). In this case, strong transcriptional activity of the BLV U3 promoter was observed when the molar ratio of cotransfected plasmid DNA to BLV proviral DNA was increased from 1:1 to 3:1. Generally, these results are in good agreement with previously reported data on activation of the BLV U3 promoter by $p38^{tax}$ in other cell lines (Willems *et al.*, 1987; Itohara *et al.*, 1988; Katoh *et al.*, 1989).

Previous reports have suggested that effective inhibition of virus replication is achieved only when 100–1000-fold molar excesses of asRNA over target RNA are used (Cotten & Birnstiel, 1989). To attain such levels, strong constitutive promoters for eukaryotic RNA polymerases II and III have been used to construct the asRNA gene (Miroshnichenko *et al.*, 1990). Our previous results have shown that asRNAs, targeted at the R-U5 region of the BLV genome and driven by the constitutive promoter of the HSV-1 TK gene were able to inhibit BLV infection in cell culture when a 10:1 molar excess of asRNA plasmid over BLV proviral DNA was used (Borisenko *et al.*, 1992). Clearly, the HSV-1 TK promoter cannot be activated by BLV-specific transactivator proteins. In the present work, we found that even the use of a 2:1 molar ratio of cotransfected pLU plasmid (containing the BLV U3 promoter-driven asRNA gene) to BLV proviral DNA resulted in 50% inhibition of virus replication in CC81 cells; with the use of a 5:1 molar ratio the inhibition of virus replication reached 75%. A significant decrease in the amount of viral genomic RNA in transfected cells was also observed when a 6:1 or 10:1 molar excess of asRNA-encoding plasmid was cotransfected with BLV proviral DNA. Paradoxically, any further increase in the molar ratio of the asRNA component impaired the antiviral effects. Moreover, we have found that the vector plasmid pLT, containing the 'empty' BLV U3 promoter without any asRNA-coding gene, also has a significant inhibitory effect on virus replication when cotransfected with BLV proviral DNA at high molar excesses. We suggested that the mechanism of inhibition of virus replication in this case might be different from that of pLU.

One possible explanation is that pLU and pLT exhibit different patterns of inhibition of BLV replication, with $p38^{tax}$ produced by the replicating BLV causing transcription of the asRNA from the first plasmid, and pLT playing a passive role as a trap for the viral transactivator. In fact, here we have demonstrated that additional expression of the BLV transactivator protein $p38^{tax}$ from the pSGtax plasmid in transfected cells totally abolishes the inhibitory effect of pLT (Fig. 6). Inasmuch as binding of BLV $p38^{tax}$ protein to a site in the BLV U3 promoter had not yet been shown, we demonstrated that this transactivator associates with the CRE/ATF responsive element. Similarly to HTLV-I $p40^{tax}$ (Marriott *et al.*, 1989), binding of BLV $p38^{tax}$ to its responsive element requires some host nuclear factors, since $p38^{tax}$ cannot interact with DNA directly (Willems *et al.*, 1992; present paper, Fig. 7). So, we concluded that at low molar excesses (1:1 to 5:1) expression of the asRNA gene causes highly efficient inhibition of BLV replication (Fig. 2, curve *a*), and the trapping effect does not play a significant role. By contrast, at high molar excesses (10:1 and greater) the transactivator trapping mechanism becomes the prevailing factor in virus inhibition. In the intermediate range, where 5:1 to 10:1 molar excesses of asRNA gene-encoding plasmid over BLV proviral DNA were used in cotransfection experiments, both mechanisms play an equal role.

Marked inhibition of virus replication by plasmid containing an empty BLV U3 promoter suggests that trapping of transactivator proteins *in vivo* can significantly decrease the concentration of transcriptional components which are necessary for productive viral infection. Studies on HTLV-I (Zhao & Giam, 1992) have shown that the concentration of $p40^{tax}$ early in replication is very low. Therefore, in transgenic animals it should be feasible to achieve competitive inhibition of virus transcription by transactivator trapping.

Since many types of viruses encode special transactivators for enhanced transcription from viral promoters, the idea of designing asRNA genes controlled by cognate viral promoters can be applied widely (Tikchonenko *et al.*, 1991). At present, the production of transgenic rabbits with asRNA genes driven by the BLV U3 promoter or with cassettes containing an empty copy of the U3 promoter is in progress. Another possibility would be to inhibit BLV infection by short DNA oligonucleotides, which can also trap viral transactivator proteins.

We thank L. Willems for the generous gift of the pSGtax plasmid, the $p38^{tax}$ protein and anti-$p38^{tax}$ monoclonal antibodies.

This work was supported in part by grants to T.T. from the Russian Fund for Basic Research (94-04-11.510-a), the Russian Fund for Gene Therapy (No. 08.03.02.01) and from the International Science Foundation (MP 2000 and MP 2300).

References

Ausubel, F. M., Breut, R., Kingston, R. E., Moore, D. D., Seidman, J. G., Smith, J. A. & Struhl, K. (1991). *Current Protocols in Molecular Biology*. New York: John Wiley.

Baurin, V., Miroshnichenko, O. I., Zakharchenko, V. I., Prokofiev, M. I., Ernst, L. K. & Tikchonenko, T. I. (1995). Transgenic rabbits with the asRNA against the RU5 LTR region of bovine leukemia virus. In *Bioengineering Farm Animals*, Part 1, pp. 154–165. Moscow. (In Russian.)

Borisenko, A. S., Miroshnichenko, O. I. & Tikchonenko, T. I. (1992). Inhibition of bovine leukaemia virus replication by the antisense RNA in cell line CC81. *Virus Research* **23**, 89–97.

Colbere-Garapin, F., Horodniceanu, F., Kourilsky, Ph. & Garapin, A.-G. (1981). A new dominant hybrid selective marker for higher eucaryotic cells. *Journal of Molecular Biology* **150**, 1–14.

Coleman, J., Hirashima, A., Inokuchi, Y., Green, P. J. & Inouye, M. (1985). A novel immune system against bacteriophage infection by using anti-sense RNA (mic RNA). *Nature* **315**, 601–603.

Cotten, M. & Birnstiel, M. L. (1989). Ribozyme mediated destruction of RNA in vivo. *EMBO Journal* **8**, 3861–3866.

Derse, D. (1988). *Trans*-acting regulation of bovine leukemia virus mRNA processing. *Journal of Virology* **61**, 1115–1119.

Ferrer, J. F., Cabradilla, C. & Gupta, P. (1981). Use of a feline cell line in the syncytia infectivity assay for detection of bovine leukemia virus infection in cattle. *American Journal of Veterinary Research* **42**, 9–14.

Graham, F. & van der Eb, A. J. (1973). A new technique for the assay of infectivity of human adenovirus 5 DNA. Virology **52**, 456–467.

Hirai, H., Fujisawa, J., Suzuki, T., Ueda, K., Muramatsu, M., Tsuboi, A., Arai, N. & Yoshida, M. (1992). Transcriptional activator Tax of HTLV-I binds to NF-kappa B precursor p105. *Oncogene* **7**, 1737–1742.

Innis, M. I., Gelfant, D. H., Sninsky, J. J. & White, T. J. (1990). *PCR Protocols: A Guide to Methods and Applications*. San Diego: Academic Press.

Itohara, S. & Sekikawa, K. (1987). Molecular cloning of infectious proviral genomes of bovine leukemia virus. *Virology* **159**, 158–160.

Itohara, S., Tomiyama, T., Ushimi, C. & Sekikawa, K. (1988). Cooperative regulation of bovine leukemia virus gene expression by two overlapping open reading frames in the XBL region. *Journal of General Virology* **69**, 797–804.

Kakidani, H., Watarai, S., Onuma, M., Tomochika, K. & Yasuda, T. (1993). Suppressive effect of liposomes containing DNA coding for diphtheria toxin A-chain on cells transformed with bovine leukemia virus. *Microbiology and Immunology* **37**, 713–720.

Katoh, I., Yoshinaka, Y., Sagata, N. & Ikawa, Y. (1987). The bovine leukemia virus X region encodes a trans-activator of its long terminal repeat. *Japanese Journal of Cancer Research* **78**, 93–98.

Katoh, I., Yoshinaka, Y. & Ikawa, Y. (1989). Bovine leukemia virus *trans*-activator $p38^{tax}$ activates heterologous promoters with a common sequence known as a cAMP-responsive element or the binding site of a cellular transcription factor ATF. *EMBO Journal* **8**, 497–503.

Kiss-Toth, E., Paca-Uccaralertkun, S., Unk, I. & Boros, I. (1993). Members of the CREB/ATF protein family, but not CREB alpha play an active role in BLV tax trans-action in vivo. *Nucleic Acids Research* **21**, 3677–3682.

Kozireva, S., Konicheva, V., Murovska, M., Baurin, V., Shayakhmetov, D., Ernst, L., Prokofiev, M. & Tikchonenko, T. (1996). Investigation of an antisense RNA gene effect on the reproduction of the bovine leukemia virus in vivo. *Transgenics* **2**, 99–109.

Marriott, S. J., Boros, I., Duvall, J. F. & Brady, J. N. (1989). Indirect binding of human T-cell leukemia virus type I tax_1 to a responsive element in the viral long terminal repeat. *Molecular and Cellular Biology* **9**, 4152–4160.

Miroshnichenko, O. I., Borisenko, A. S., Ponomareva, T. I. & Tikchonenko, T. I. (1990). Inhibition of adenovirus replication by the E1A antisense transcript initiated from hsp70 and VA-1 promoters. *Biomedical Science* **1**, 267–273.

Orita, S., Sato, S., Aono, Y., Minoura, N., Yamashita, T., Hinuma, Y. & Igarashi, H. (1993). Identification of novel singly spliced pX mRNA transcripts common to all human T-cell leukemia virus type 1-related retroviruses. *Virus Genes* **7**, 197–204.

Powers, M. A. & Radke, K. (1992). Activation of bovine leukemia virus transcription in lymphocytes from infected sheep: rapid transition through early to late gene expression. *Journal of Virology* **66**, 4769–4777.

Rhodes, A. & James, W. (1990). Inhibition of human immunodeficiency virus replication in cell culture by endogenously synthesized antisense RNA. *Journal of General Virology* **71**, 1965–1974.

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. *Proceedings of the National Academy of Sciences, USA* **82**, 677–681.

Sambrook, J., Fritsch, E. F. & Maniatis, T. (1989). *Molecular Cloning: A Laboratory Manual*, 2nd edn. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.

Takayama, K. M., Kuriyama, S., Weiss, S., Chada, K., Inouye, S. & Inouye, M. (1989). Antisense RNA-mediated inhibition of viral infection in tissue culture and transgenic mice. In *Molecular Biology of RNA*, pp. 299–310. New York: Alan R. Liss.

Tikchonenko, T. I. (1991). Antisense polynucleotides as factor of informational immunity. In *Biotechnology on the Threshold of XXI Century*, pp. 68–71. Moscow: Russian Academy of Sciences.

Wang, S. J. & Dolnick, B. J. (1993). Quantitative evaluation of intracellular sense–antisense RNA hybrid duplexes. *Nucleic Acids Research* **21**, 4383–4391.

Willems, L., Gegonne, A., Chen, G., Burny, A., Kettmann, R. & Ghysdael, J. (1987). The bovine leukemia virus p34 is a transactivator protein. *EMBO Journal* **6**, 3385–3389.

Willems, L., Kettmann, R., Chen, G., Portetelle, D., Burny, A. & Derse, D. (1992). A cyclic AMP-responsive DNA-binding protein (CREB2) is a cellular transactivator of bovine leukemia virus long terminal repeat. *Journal of Virology* **66**, 766–772.

Zhao, L.-J. & Giam, C.-Z. (1992). Human T-cell lymphotropic virus type I (HTLV-I) transcriptional activator Tax enhances CREB binding to HTLV-I 21-base-pair repeats by protein–protein interaction. *Proceedings of the National Academy of Sciences, USA* **89**, 7070–7074.

Received 17 December 1996; Accepted 19 March 1997