Solitary HERV-K LTRs possess bi-directional promoter activity and contain a negative regulatory element in the U5 region

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Abstract Reporter gene analysis of HERV-K solitary long terminal repeats (LTRs) showed that they retain detectable activity in human teratocarcinoma cells, and can direct the transcription in both orientations relative to the reporter gene. Deletion analysis demonstrated the possible existence of alternative promoters within the LTR as well as a silencer-like element in the U5 region. Our results indicate also that all-*trans*-retinoic acid is capable of modulating expression of the reporter gene directed by a HERV-K LTR in NT2/D1 cells.

Key words: Human endogenous retrovirus; HERV-K; Long terminal repeat; Regulation of transcription

1. Introduction

At least 1% of the human genome is occupied by endogenous retroviral (ERV) sequences. Most of the human ERVs (HERVs) sequenced to date represent defective proviruses. However, various human cell lines and tissues were found to express mRNAs or even proteins encoded by HERVs [1–3]. The HERV gene expression is mostly under control of their LTRs, which harbour multiple signals, recognised by cellular transcriptional machinery [4]. By virtue of their ability to integrate randomly into the host genome and alter expression of nearby genes, the ERVs represent an important class of insertion mutagens, which may also provide new regulatory elements for linked DNA sequences [5]. Occasional homologous recombination between the LTRs results in the excision of the provirus genome and generation of solitary LTR, which can retain the whole set of the regulatory signals. In this way, a large number of solitary HERV LTRs has been created during evolution. One of the most important tasks of functional genomics is to reveal the constituents and arrangement of the transcriptional regulation network. Therefore the regulatory potential and abundance of HERV LTRs in the genome are a great challenge posing many questions as to the impact of these elements on the genome functioning. The first step towards the understanding of the HERV LTRs-genome interplay should involve comprehensive analysis of the functional properties of endogenous LTRs that resided in the genome over millions years of evolution. In particular, we have recently observed a frequent proximity of solitary HERV-K LTRs and genes on human chromosome 19 [6,7]. Moreover, specific interactions of the HERV-K LTRs with nuclear proteins from different human cells were also demonstrated [8,9]. Though the data provide the basis for the speculations concerning the possible involvement of these solitary HERV-K LTRs in the neighbouring gene regulation, however, there is no direct evidence to support this view. One way to investigate regulatory properties of solitary LTRs is to examine their abilities to activate a promoterless reporter gene [10]. Using this approach we have revealed and describe in this report three functional features of solitary HERV-K LTRs; (i) they retain considerable promoter and probably enhancer potential; (ii) they can function as promoters in both orientations relative to the reporter gene and (iii) they contain a negative regulatory element in their U5 region suppressing their promoter activity.

2. Materials and methods

2.1. Cloning of LTR fragments

HERV-K LTRs were isolated as polymerase chain reaction (PCR) fragments from 23280, 24321 and 29471 LLNL (Lawrence Livermore National Laboratory, CA, USA) cosmid clones as described previously [6,11]. Below these LTRs will be referred to as LTR23280, LTR24321 and LTR29471, respectively. The primers 5′-CAGTCTTATCTCCTTTAACTGACCA-3′ (Prl), 5′-CTCGTGTTTGTGCTTGG-3′ (Pr2), corresponding to the LTR23280 attached regions and LTR-specific primers 5′-GAGATCAGATTGTTACTGTGTC-3′ (Pr3), 5′-ATTGTCCAAGGTTTCTCCCC-3′ (Pr4), 5′-GCCTTAGGGCTGGAGGTG-3′ (Pr5) and 5′-AAAGACACAGAGACAAAGTATAGA-3′ (Pr6) were used for PCR amplification of full-sized LTRs and their fragments. Amplification of the LTRs was performed using native *Pfu* DNA polymerase (Stratagene) in standard PCR conditions recommended by the manufacturer. Gel purified PCR fragments were cloned in *Sma*I digested promoterless pGL-3 Basic Vector (Promega). Plasmids pGLT47D were constructed by subcloning of *Bam*HI–*Hin*dII fragment containing 5′HERV-K-T47D LTR [14] into pGL3 Basic Vector (Promega).

2.2. Cell culture

The human teratocarcinoma cell line NT2/D1 (ATCC CRL-1973) was cultured at 37°C in DMEM/RPMI 1640 (1:1) medium mixture supplemented with 10% foetal calf serum (FCS), 2 mM glutamine and antibiotics. To induce differentiation, NT2/D1 cells were cultured in the presence of 10 μM all-*trans*-retinoic acid (RA, Sigma).

2.3. Transfections and luciferase assay

Transient transfections were performed by the DEAE-dextran method [12] in subconfluent 25 cm^2 flasks. Each flask was transfected with 10 μg of the luciferase expression plasmid and 1 μg of pCMV-LacZ plasmid as an internal control. Cells were maintained for 48 h in DMEM/RPMI 1640 (1:1) medium with 10% FCS and then harvested

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for luciferase assay (Promega) and ONPG assay for β-galactosidase activity [13].

Stable transfections were performed by lipofectin-mediated method in 75 cm^2 flask with 2×10^6 NT2/D1 cells per flask. The cells were washed twice with serum-free Opti-MEM (Gibco-BRL) followed by the replacement of the medium with Opti-MEM containing 4 μg of the luciferase expression plasmid, 1 μg of pSV2Neo co-transfected plasmid and 10 μl of lipofectin (Gibco-BRL) at 2 ml per flask. The cells were incubated with lipofectin-DNA mix for 5–6 h and then cultured for 2 days after addition of 10 ml of DMEM/RPMI 1640 (1:1) medium containing 10% FCS and lacking antibiotics. Neomycin-resistant clones were further selected by incubation in the medium containing 1 mg/ml G418 (Gibco-BRL) for 2 weeks. Non-cloned cell populations were harvested for luciferase assay. All cell lines were derived from at least 100 separate colonies.

The total protein in each sample was determined by BCA reagent (Pierce), and luciferase activity was normalised to the total protein in each sample. All transfections were carried out in at least three independent experiments using different preparations of the plasmids.

3. Results and discussion

To characterise the possible promoter activity of solitary

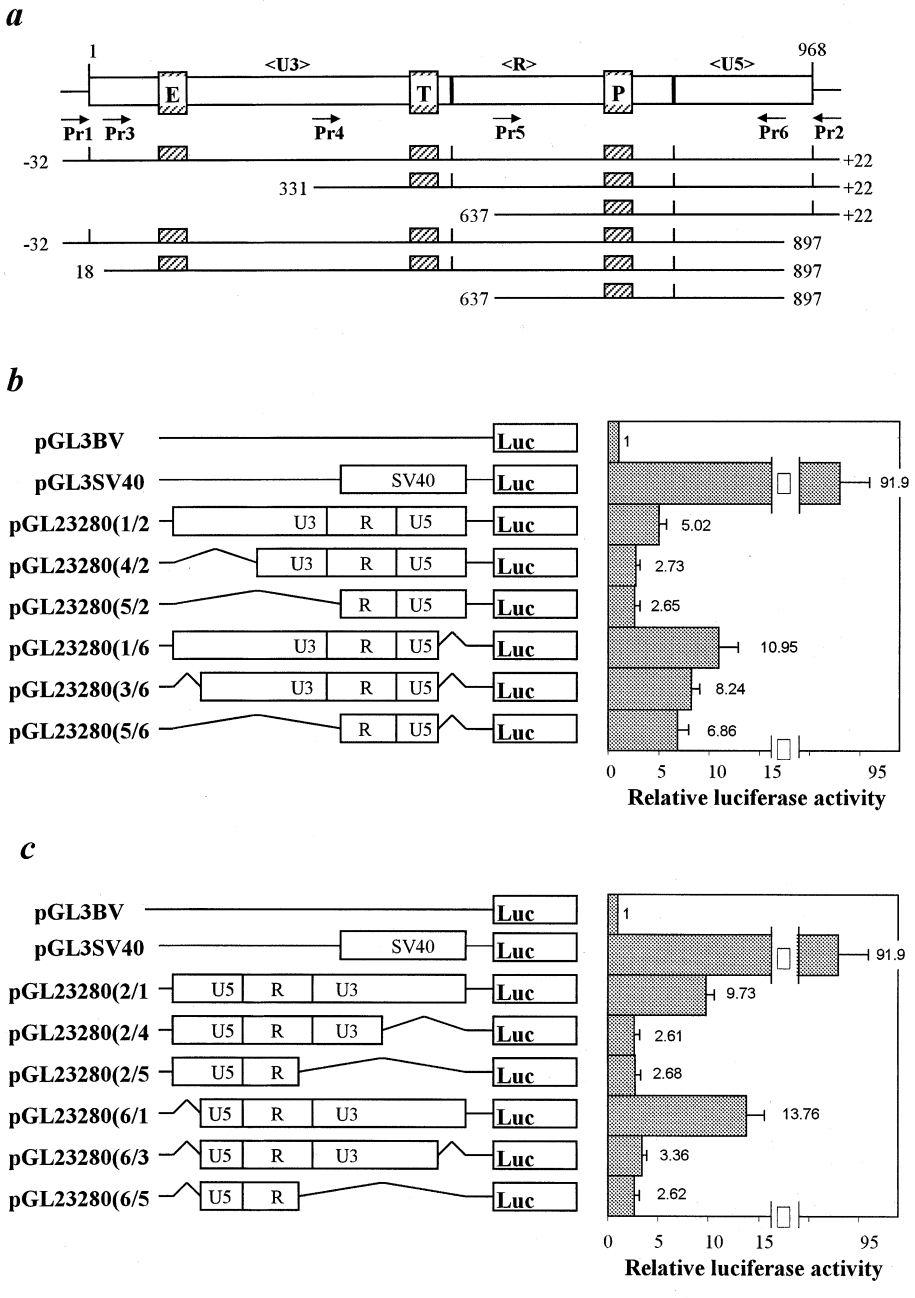

Fig. 1. Regulation of luciferase reporter gene activity by solitary full-length HERV-K LTR23280 and its fragments. a: Schematic diagram of the solitary HERV-K LTR 23280 (GenBank accession number L47334) and corresponding PCR fragments of LTR23280. The arrows labelled Pr1, Pr2, Pr3, Pr4, Pr5 and Pr6 indicate the locations of primers used in PCR. The locations of the enhancer region (E), consensus TATA box (T), and consensus polyadenylation signal (P) are indicated as hatched boxes. The ends of PCR fragments are indicated by their positions (in bp) at the solitary LTR. Luciferase assay for LTR23280 constructs with positive-sense (b) or negative-sense (c) orientation of the LTR fragments. Results are expressed as ratios of the luciferase activity to that of the promoterless pGL3BV reporter plasmid. The average and standard error of the mean (error bars) are presented. All transfections were carried out in three to six independent experiments.

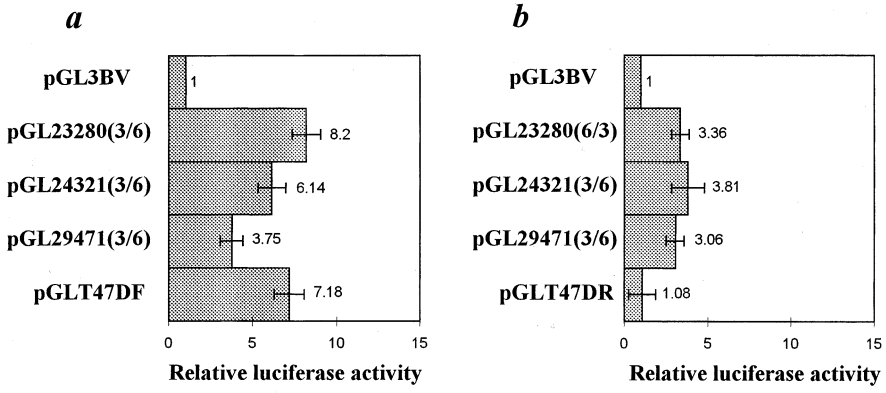

Fig. 2. Assay of HERV-K LTR promoter activity in transient transfected NT2/D1 cells. LTRs 23280, 24321, and 29471 were obtained by PCR amplification using Pr3 and Pr6 primers and DNAs from corresponding LLNL cosmid clones [6]. The LTR fragments were cloned into pGL3BV reporter plasmid in forward (a) and reverse (b) orientation and LTR luciferase gene constructs were used to transfect NT2/D1 cells. Results are expressed as ratios of the luciferase activity to that of the promoterless pGL3BV reporter plasmid. The average and standard error of the mean (error bars) are presented. All transfections were carried out in at least five independent experiments.

HERV-K LTRs, we analysed the ability of one of them, LTR23280, to drive expression of luciferase gene after cloning into promoterless pGL3 vector. The full-sized LTR23280 was obtained by PCR using cosmid DNA as the template and Pr1/Pr2 pair of primers (Fig. 1A). The resulting plasmid pGL23280(1/2) containing the full-sized LTR23280 in forward orientation relative to the reporter gene was transfected into NT2/D1 cells, and the promoter activity of the LTR was measured by the luciferase activity compared with the activity of the extract from the promoterless pGL3-transfected cells (Fig. 1B). The results clearly indicate that LTR23280 is capable to drive luciferase gene expression in NT2/D1 cells. A reporter construct pGL23280(2/1) containing the full-sized LTR23280 in reverse orientation towards the reporter gene was tested in a parallel transfection assay (Fig. 1C). Unexpectedly, the luciferase expression was enhanced also in this case thus demonstrating bi-directional promoter activity of LTR23280. Its promoter 'strength' was approximately 6% ('forward' promoter) and 10% ('reverse' promoter) of the SV40 promoter in NT2/D1 cells. A pGL3 vector lacks any eukaryotic promoter or enhancer and contains specific sequences to prevent transcription from any cryptic promoter located elsewhere in the plasmid as specified by the vendor. Nevertheless, we reproduced the results using another promoterless reporter pBV vector [14], thus confirming that the luciferase expression in transfected cells was the result of the LTR promoter activity and not the plasmid cryptic promoter activity boosted by the LTR enhancer. Until recently the ERV LTR promoter activity was studied mainly for 'natural' forward orientation relative to the provirus genes, though the reverse promoter activity of the U3 region of solitary HERV-H LTRs [10] and murine IAP LTRs [15] has also been reported. Moreover, the SP1-dependent negative-sense reverse promoter of HIV-1 LTRs was recently demonstrated [16]. Our finding suggest that at least some of solitary HERV-K LTRs are capable of promoting transcription of downstream as well as upstream genes. Possible function of reverse promoter in the ERV LTRs may include involvement in inhibition of flanking cellular gene transcription, production of double-stranded RNA or the regulation of synthesis of pos-

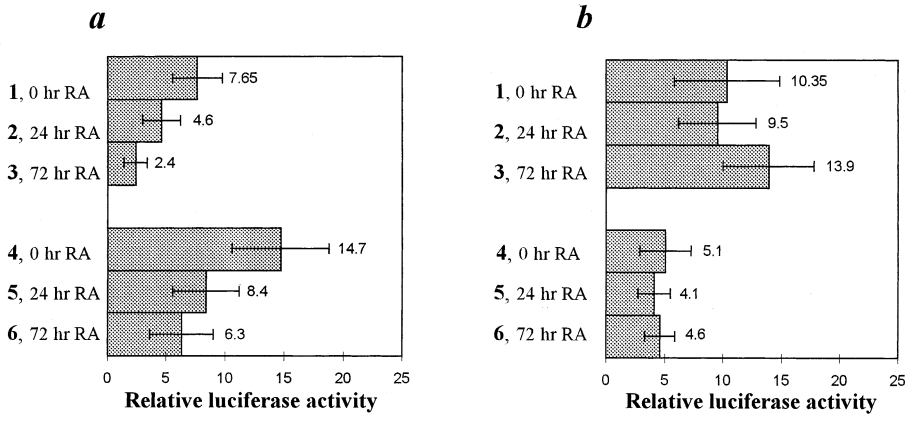

Fig. 3. Assay of HERV-K LTR promoter activity in stably transfected NT2/D1 cells. The plasmids pGL23280 (1.2) (**a1, a2, and a3**), pGL23280(2/1) (**b1, b2, and b3**), pGL23280(3/6) (**a4, a5 and a6**), and pGL23280(6/3) (**b4, b5 and b6**) were used for stable transfections of NT2/D1 cells. To induce differentiation NT2/D1 were grown for 24 or 72 h in the presence 10 μM RA. The RA-treated cells and non-treated controls (0 h RA) were harvested and levels of luciferase activities in cell extracts were determined. Results are expressed as ratios of the luciferase activity to that of the promoterless pGL3BV reporter plasmid. The average and standard error of the mean (error bars) are presented. The stable transfections were carried out in two independent experiments.

itive-strand-derived proteins by antisense RNA. The human genome contains more than 10 000 solitary HERV-K LTRs [17,18], and the fact of bi-directional transcription initiated from these abundant and dispersed sequences adds a new feature to their functional interactions with other genomic elements. It should be noted that such a bi-directional activity is however not an attribute of all HERV-K LTRs: whereas all HERV-K LTRs (HML-2 subfamily) tested revealed different levels of such a 'bi-directionalism', a representative of another LTR subfamily (HML-6)-5′LTR of HERV-K-T47D was active only in the forward orientation relative to the reporter gene (Fig. 2).

We have previously reported that some of solitary HERV-K (HML-2) LTRs in the human genome are altered by deletions in their 5′- and 3′-flanking regions [6,11]. To examine the impact of these deletions on the HERV-K LTR promoter activity we constructed reporter plasmids containing LTR23280 bearing various deletions in its 5′- and 3′-flanking regions (Fig. 1B,C). Irrespective of its orientation relative to the luciferase gene, a truncated artificial LTR with the 'enhancer' region deleted had a considerable (about two-fold) decreased promoter activity. It suggests that the deleted region be characterised by some enhancer-like activity. Interestingly, this region was also shown to specifically interact with nuclear proteins [8]. The further trimming of the LTR up to the removal of the putative TATA box did not considerably affect the promoter activity as compared to the 'enhancer' region deletion. Another finding of this study was an unexpected detectable promoter activity in the forward orientation of a U5 deleted LTR23280 completely lacking the U3 region (pGL23280(5/6) construct). These suggest that a HERV-K LTR may contain an alternative promoter independent on the TATA box and located downstream of the U3/R boundary. Studies to sequence and specificity of this R/U5 promoter of LTR23280 are under way. An unexpected effect was observed when a 71-bp long fragment of the U5 region had been deleted. The so truncated LTR was considerably more active than its full-sized analogue independent of orientation. The deleted fragment probably contained a negative silencer-like regulatory element suppressing the LTR promoter activity [19]. Similar inhibitory effects of some structural elements in the U5 regions were described for human foamy virus [20] and for T-cell leukemia virus type 1 [21,22]. This similarity with complex retroviruses is in line with recently detected functional homology of the Rev protein and a sequence-specific nuclear RNA export factor, termed K-Rev, encoded within a HERV-K LTR [23,24].

A sequence analysis of HERV-K LTRs allowed us earlier to classify most of the solitary HERV-K LTRs into several subgroups that appeared in the primate genomes at different times of the evolution [7]. According of this classification, LTR 23280 is a typical 'young' HERV-K LTR with the estimated time of emergence in the human genome of 5–6 millions years (Myr) ago. To find out whether a most ancient LTR29471 integrated in the human genome as early as 42 Myr ago retained its promoter activity we also tested it as described above. The results are presented in Fig. 2. In the same series of experiments another 'young' LTR24321 (5 Myr) was also analysed. Pr1/PR2 pair of primers was used for PCR amplification of nearly full-sized LTR fragments deprived of the presumed negative regulator, which were subsequently cloned into promoterless pGL3 vector in both orientations. The results of the transfection clearly indicate that both 'young' LTRs (23280 and 24321) as well as an 'old' LTR 23471 are capable to direct the luciferase expression in NT2/D1 cells, although their individual promoter strengths are different (Fig. 2). The 'forward' promoter activity of 'old' LTR29471 was lower than that of 'young' LTRs 23280 and 24321. All these three solitary HERV-K LTRs showed detectable and close-promoter activities also in the reverse orientation (Fig. 2B).

The solitary HERV LTRs are integral constituents of chromosomal DNA. The transient expression of extrachromosomal reporter constructs containing LTR promoter does not exactly simulate the LTR functioning under real physiological conditions. Therefore we confirmed the conclusions deduced from the transient expression analysis by stable transfection of NT2/D1 cells with HERV-K reporter gene constructs (Fig. 3). With these stable transformants we also investigated the dependence of LTR directed luciferase expression on differentiation status of the cells. Since RA has been shown to induce NT2/D1 cell differentiation into postmitotic neurones [25], we investigate the effect of RA treatment on expression of LTR 23280 luciferase reporter constructs. The data presented in Fig. 3A demonstrated that 'forward' promoter activity of this HERV-K LTR constantly decreased with time after the RA addition to the medium. At that time RA did not affect the 'reverse' LTR promoter activity (Fig. 3B). The results show that the HERV-K LTR activity in its direct orientation may depend on the differentiation status of the cells.

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References

[1] Hohenadl, C., Leib-Mosch, C., Hehlmann, R. and Erfle, V. (1996) J. Acquired Immune Defic. Syndr. Hum. Retrovirol. 13, 268–273.
[2] Lower, R., Lower, J. and Kurth, R. (1996) Proc. Natl. Acad. Sci. USA 93, 5177–5184.
[3] Larsson, E. and Andersson, G. (1998) Scand. J. Immunol. 48, 329–338.
[4] Coffin, J.M. (1996) in: Fields Virology (Fields, B.N., Ed.), pp. 1767–1847, Lippincott-Raven, Philadelphia, PA.
[5] Di Cristofano, A., Strazullo, M., Longo, L. and La Mantia, G. (1995) Nucleic Acids Res. 23, 2823–2830.
[6] Vinogradova, T., Volik, S., Lebedev, Yu., Shevchenko, Yu., Lavrentyeva, I., Khil, P., Grzeschik, K.H., Ashworth, L.K. and Sverdlov, E. (1997) Gene 199, 255–264.
[7] Lavrentieva, I., Khil, P., Vinogradova, T., Akhmedov, A., Lapuk, A., Shakhova, O., Lebedev, Y., Monastyrskaya, G. and Sverdlov, E.D. (1998) Hum. Genet. 102, 107–116.
[8] Akopov, S.B., Nikolaev, L.G., Khil, P.P., Lebedev, Y.B. and Sverdlov, E.D. (1998) FEBS Lett. 421, 229–233.
[9] Knossl, M., Lower, R. and Lower, J. (1999) J. Virol. 73, 1254–1261.
[10] Feuchter, A. and Mager, D. (1990) Nucleic Acids Res. 18, 1261–1270.
[11] Lebedev, Y.B., Volik, S.V., Obradovic, D., Ermolaeva, O.D., Ashworth, L.K., Lennon, G.G. and Sverdlov, E.D. (1995) Mol. Gen. Genet. 247, 742–748.
[12] McCutchan, J.H. and Pagano, J.S. (1968) J. Natl. Cancer Inst. 41, 351–357.
[13] Miller, J.H. (1972) Experiments in molecular genetics, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
[14] Seifarth, W., Baust, C., Murr, A., Skladny, H., Krieg-Schneider,

F., Blusch, J., Werner, T., Hehlmann, R. and Leib-Mosch, C. (1998) J. Virol. 72, 8384–8391.
[15] Christy, R.J. and Huang, R.C. (1988) Mol. Cell. Biol. 8, 1093–1102.
[16] Peeters, A., Lambert, P.F. and Deacon, N.J. (1996) J. Virol. 70, 6665–6672.
[17] Leib-Mosch, C., Haltmeier, M., Werner, T., Geigl, E.M., Brank, W.R., Franke, U., Erfle, V. and Hehlmann, R. (1993) Genomics 18, 261–269.
[18] Sverdlov, E.D. (1998) FEBS Lett. 428, 1–6.
[19] Ogbourne, S. and Antalis, T.M. (1998) Biochem. J. 331, 1–14.
[20] Yang, P., Zemba, M., Aboud, M., Flugel, R.M. and Lochelt, M. (1997) Virus Genes 15, 17–23.
[21] Seiki, M., Hikikoshi, A. and Yoshida, M. (1990) Virology 176, 81–86.
[22] Okumura, K., Sakaguchi, G., Takagi, S., Naito, K., Mimori, T. and Igarashi, H. (1996) J. Biol. Chem. 271, 12944–12950.
[23] Magin, C., Lower, R. and Lower, J. (1999) J. Virol. 73, 9496–9507.
[24] Yang, J., Bogerd, H.P., Peng, S., Wiegand, H., Truant, R. and Cullen, B.R. (1999) Proc. Natl. Acad. Sci. USA 96, 13404–13408.
[25] Pleasure, S.J., Page, C. and Lee, V.M. (1992) J. Neurosci. 12, 1802–1815.