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The RU5 ('R') region from human leukaemia viruses (HTLV-1) contains an internal ribosome entry site (IRES)-like sequence

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Abstract RNA fragments containing the complete R region and the beginning of the U5 region ('R') from the human T cell leukaemia virus 1 (HTLV-1) stimulated the translation of the second cistrons in bicistronic mRNAs. The 5′ untranslated region from SV40 early genes (SU) which was unable to stimulate translation of second cistrons amplified markedly the internal ribosome entry site (IRES) effect of the HTLV-1 'R' fragments. The 'R' regions from HTLV-1 have therefore properties similar to internal ribosome entry sites (IRES) originally found in picornavirus. The beginning of the U5 region from HTLV-1 contains a polypyrimidine sequence which is known to play an essential role in the IRES activity in picornavirus. The same experiments carried out using the 'R' region from bovine leukaemia virus (BLV) showed that this sequence has at most a weak IRES effect. One retroviruses HTLV-1 and perhaps others contain therefore an IRES activity. Interestingly, the combined SU 'R' sequence worked efficiently with different cistrons, different promoters and in all tested cell lines, whereas the poliovirus IRES was active in CHO cells but not in the mouse mammary cell line HC11. The SU 'R' sequence may therefore preferably be used to generate active bicistronic mRNAs.

Key words: Internal ribosome entry site; R region; Human T cell leukaemia virus 1; Bovine leukaemia virus

1. Introduction

The initiator of mRNA translation is a complex process involving the cap structure located in the 5′P end of the mRNA. A complex containing the 40S ribosome subunit and translation factors are formed at the cap level [1]. This complex migrates along the 5′ untranslated region (5′UTR), according to a scanning mechanism, until it meets an initiator codon preceded by the consensus sequence defined by M. Kozak [2]. In agreement with this model, it has been shown that the translation efficiency is greatly reduced when the 5′UTR contains a secondary structure [3]. This rule is, however, not respected in all cases. Indeed, in previous work, we observed that a combination of the SV40 early gene 5′UTR (SU) and the R region with the first 39 nucleotides of U5 (designated as 'R') from the human T cell leukaemia virus 1 (HTLV-1) markedly stimulates translation although it is highly structured (Attal et al., unpublished results). Another well-known exception is the case of picornavirus. The mRNA from these viruses have long and highly structured 5′UTRs which work efficiently in a cap-independent manner [4]. This concept is based on several experimental facts. The mRNA devoid of cap structure can be translated efficiently in a cell-free system when they contain in their 5′P end a picornavirus 5′UTR. In vitro and in vivo, the mRNAs having an hairpin loop in their 5′P end can be translated efficiently if they have a picornavirus 5′UTR. In cells infected by poliovirus, the viral proteinase 2A which inactivates eIF4G does not prevent translation of an mRNA which contains a picornavirus 5′UTR. It is generally admitted that the picornavirus 5′UTRs are able to bind directly ribosomes in a cap-independent manner and without a previous scanning along the mRNA. Specific protein factors are involved in this process and a polypyrimidine located in the 3′OH end of the picornavirus 5′UTRs is required [5]. The picornavirus 5′UTRs are therefore considered as containing an internal ribosome entry site (IRES).

In higher eucaryotes, most of the mRNAs are monocistronic. When a second cistron is added at the end of an mRNA, it is generally very poorly translated. However, in optimized conditions, the second cistron can be translated efficiently [6]. Interestingly, the addition of picornavirus IRES between two cistrons greatly stimulates the translation of the second [7]. This property is considered as an additional proof that picornavirus 5′UTRs contain functional IRES.

RNA sequences having IRES properties has been found in number of viruses including most, if not all, the members of the picornavirus family [8], in hepatitis C [9], murine hepatitis virus [10], pestivirus [11], Moloney mouse leukaemia virus [12] and Friend mouse leukaemia virus [13]. These structures have a more or less similar mechanism of action [14,15]. Interestingly, RNA sequences with IRES activity have also been found in several cellular mRNAs including those coding for the immunoglobulin heavy chain binding protein (BIP) [16], the antennapedia [17] and the β chain of kinesin [18]. All these sequences are considered to remain active when cells are stressed and when the translation factors cannot use any more the cap [19,20].

All the IRES seem to have in common the fact that they are highly structured. In a previous study, we observed that the R region from HTLV-1 and to a lower degree from BLV stimulate mRNA translation although they are highly structured. The effect of R from HTLV-1 was greatly amplified by the SV40 early gene 5′UTR (SU) (Attal et al., unpublished results). The present work has been undertaken to determine if R regions from both retroviruses associated or not to the 5′UTR from SV40 have an IRES activity.

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2. Materials and methods

2.1. Gene constructions

Three promoters have been used: the rabbit whey acidic gene upstream (WAP) region which is active in mammary cells, a combination of SV40 enhancer and human cytomegalovirus (CMV) promoter and the Rous sarcoma virus (RSV) promoter which work in most cell types. These regulatory regions have been used and described in our previous studies (Attal et al., unpublished results, [21]).

The 'R' region from HTLV-1 was the 267 bp DNA fragment defined by Takebe et al. [22]. It contains the whole R and a 39 nucleotides of U5 region.

The BLV R region was the a 250 bp Hae III fragment which contained only the R part of the viral genome minus a few nucleotides in the 3'OH end. It was the generous gift of Dr. L. Willems.

The poliovirus 5'UTR containing the IRES was kindly provided by Dr. N. Sonenberg.

The SV40 early gene 5'UTR (SU) contained the 86 bp following the cap site.

The human growth hormone (hGH) cDNA and the luciferase gene were used as the first cistron. The reporter genes were the bovine growth hormone (bGH) cDNA and the chloramphenicol acetyl transferase gene.

All constructs with WAP and CMV promoters contain the transcription terminator from the SV40 late genes.

The plasmid p245 was derived from pRSV luciferase plasmid [23] after cloning a sequence containing SU 'R' CAT (chloramphenicol acetyl transferase gene) between the stop codon of the luciferase gene and the transcription terminator from the SV40 early genes.

The plasmid p262 was constructed by introducing the synthetic 30 mers oligodeoxynucleotide pAGCTTGGCCGGGCGCGGCCGCG-CCCGGCCA in the *Hin*dIII site between the RSV promoter and luciferase gene. This oligonucleotide is complementary to itself and two molecules can form a double-strand DNA for cloning. On the other hand, the coding strand generates a GC-rich stable hairpin loop.

All these elements were associated using the classical method of genetic engineering. They were introduced in the plasmid pPoly III as described in our previous studies (Attal et al., unpublished results, [21]).

All the gene constructs used are described in Fig. 1.

2.2. Cell transfection and protein measurements

The mouse mammary cells HC11 were transfected at mid-confluency using lipofectamine (BRL). The plasmid pRSV neo was added to the plasmids of interest containing the WAP gene promoter to obtain stable clones. The mixed clones were cultured to hyperconfluency in the presence of fetal calf serum and EGF (10 ng/ml) and induced by insulin (5 μg/ml), dexamethasone (10^{-6} M) and ovine prolactin (NIH-PS13) (5 μg/ml) for 2 days in the absence of EGF and serum as described previously (Attal et al., unpublished results, [21]). Culture medium were collected at the end of the induction and bGH was measured using a specific radioimmunoassay (Attal et al., unpublished results, [21]).

CHO cells were transfected with the plasmids containing the CMV and RSV promoters using lipofectamine. Two days after transfection the medium was collected for bGH measurements, the CHO cells were scrapped for luciferase and CAT analysis. Luciferase activity was measured according technique published by De Wet et al. [23] in a Lumat LB9501 (Berthold, Wildbad, Germany). Cellular extracts were assayed for CAT activity using a mixed phase assay [24].

2.3. Northern blot analysis

RNA isolation and Northern blot analysis were performed as previously described [25].

3. Results

The bicistronic mRNA containing only hGH and bGH cDNAs (p9') and the WAP gene promoter was poorly capable of translating the bGH cistron in HC11 cells (Fig. 2). The addition of the R region from HTLV-1 (p202) enhanced quite significantly the translation of the bGH cistron. The R region from BLV (p203') showed a lower stimulatory effect. The

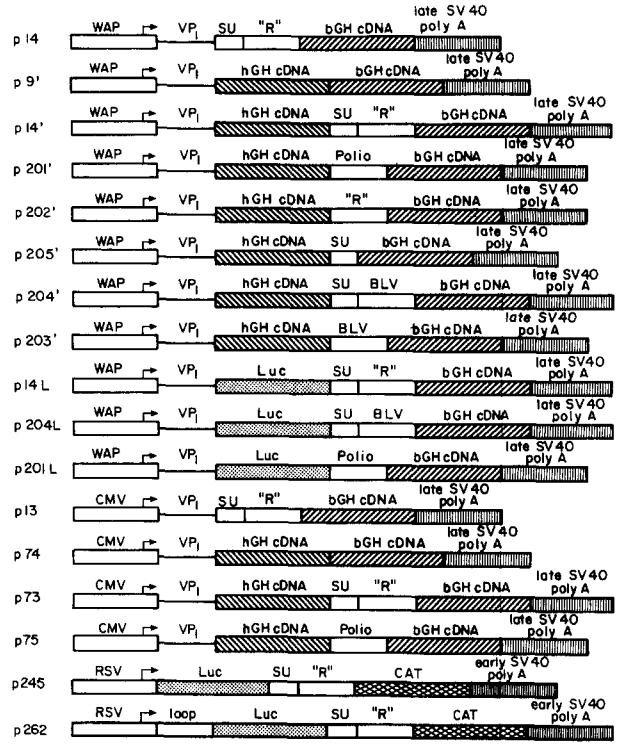

Fig. 1. Schematic representation of the gene constructions. The different elements are not on scale. WAP: rabbit whey acidic promoter; SU: SV40 early gene 5; UTR; bGH bovine growth hormone; cDNA hGH: human growth hormone cDNA; VP1: intron from the SV40 late genes; late SV40 polyA: transcription terminateur from SV40 late genes; R: the 267 bp from HTLV-1 containing the R region and 39 bp of U5; Polio: IRES from poliovirus; BLV: the R region minus a few nucleotides in the 3'0H from the bovine leukaemia virus; CMV: human cyto-megalovirus promoter; SV40 enhancer; Luc: firefly luciferase gene.

SV40 early gene 5'UTR (SU) was totally inactive alone (p205'). This RNA sequence markedly amplified the action of the R region from HTLV-1 (p14') but not from BLV (p204'). Interestingly, the combination SU 'R' allowed translation of the bGH cistron at a level as high as 70% of that obtained with monocistronic mRNA (p14). Surprisingly, the poliovirus IRES (p201') was almost completely inactive in these conditions.

In order to determine if the IRES activity of the R regions from HTLV-1 and BLV was dependent on the nature of the first cistron, the hGH cDNA was replaced by the firefly luciferase gene. Results shown in Fig. 3 confirm that the SU 'R' and to a lower degree SU BLV associations were able to promote the translation of the bGH cistron. The poliovirus IRES showed only a very weak activity as in the case of the p201' construct. This lack of bGH synthesis was not due to the absence of the mRNA since the luciferase activity in the cellular extract was quite high (not shown).

Unexpectedly, the bGH cistron preceded by the SU 'R' combination (p14L) was translated at a level representing only 20% of the corresponding monocistronic mRNA (p14). This suggests that both cistrons work in a non-totally independent manner, even in the presence of the IRES.

In order to determine if the cell type may favour more or less the activity of IRES, genes containing the hGH and the bGH cistrons, the hCMV promoter and the different IRES

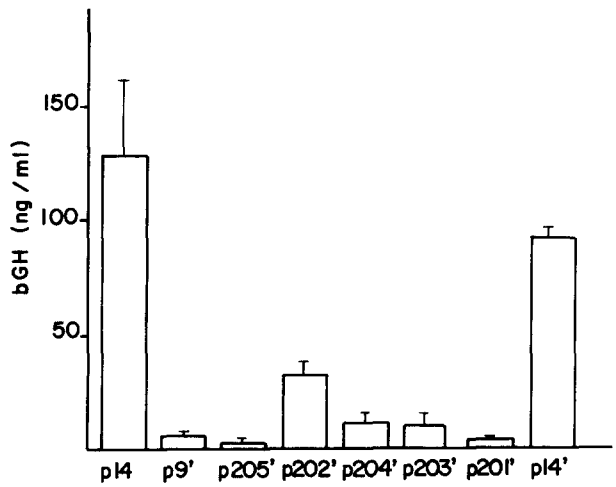

Fig. 2. The IRES effect of the R region from HTLV-1 and BLV. Bicistronic constructs containing the bGH and hGH cDNAs and the WAP gene promoter were use to generate stable HC11 clone, bGH was measured in the medium after hormonal induction. The plasmids used are those depicted in Fig. 1.

were constructed and transfected into CHO cells. Results shown in Fig. 4 indicate with no ambiguity that the bicistronic mRNA devoid of IRES (p74) was unable to translate the bGH cistron. The SU 'R' IRES (p73) was quite efficient, leading to a bGH synthesis representing about 60% of the corresponding monocistronic mRNA (p13). The poliovirus IRES (p75) was of similar efficiency.

The SU 'R' was also used in several other combinations of cistrons (firefly luciferase, β-galactosidase, chloramphenicol acetyl transferase, ...). It was quite efficient to express the second coding region in transfected cultured cells and in transgenic mice and rabbits (not shown).

In addition to bGH in the culture medium, a luciferase activity was found in the extracts from cells transfected with plasmids containing the corresponding cistron in the first position. To make sure that the SU 'R' sequence between the two cistrons did not act as a cryptic promoter, CHO cells were transfected with plasmid p73. The RNA was extracted from the cells. Northern blot analysis using bGH and hGH cDNA as probes shown in Fig. 5 indicates with no ambiguity that a single mRNA containing 2.2 kb was obtained after transfection with the plasmid p73. Moreover, experiments not shown here indicated that the SU 'R' sequence enhanced at most marginally the concentration of the corresponding mono and bicistronic mRNA in CHO cells.

In previous studies, it has been shown that in biscistronic mRNA containing an IRES, translation of the first cistron is severely reduced by the addition of a stable hairpin loop after the cap site whereas translation of the second cistron is not affected. The hairpin loop described in Section 2 was able to reduced the expression of monocistronic mRNA to 15–20% of the control (not shown). The same hairpin loop added in the plasmid p245 (Fig. 1) before the luciferase cistron (p262) attenuated quite significantly its expression but it did not modify CAT activity (Fig. 6). One possible interpretation of these

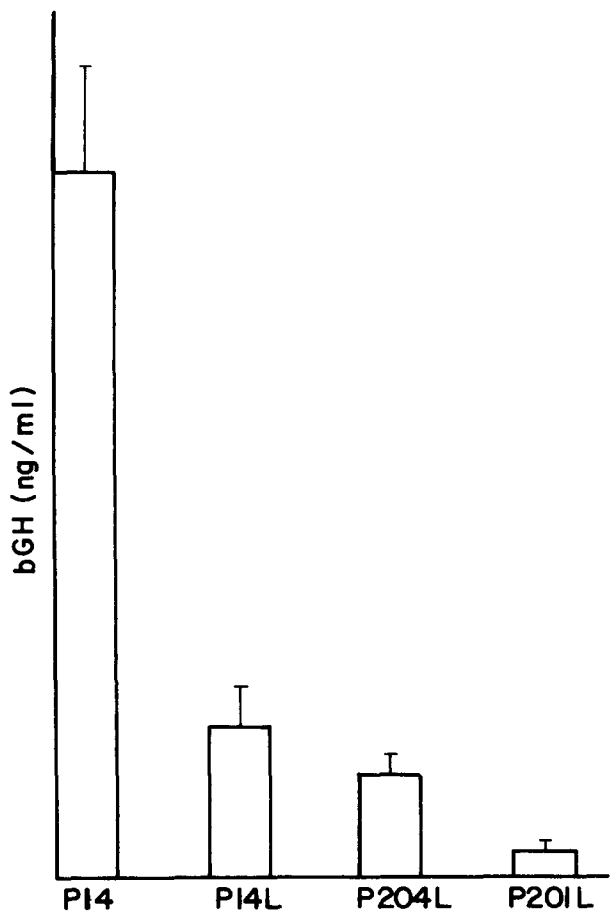

Fig. 3. The IRES effect of the R regions from HTLV-1 and BLV in HC11 cells. The bicistronic constructs contained the luciferase and the bGH cDNAs driven by the WAP gene promoter. bGH was measured in the medium after hormonal induction.

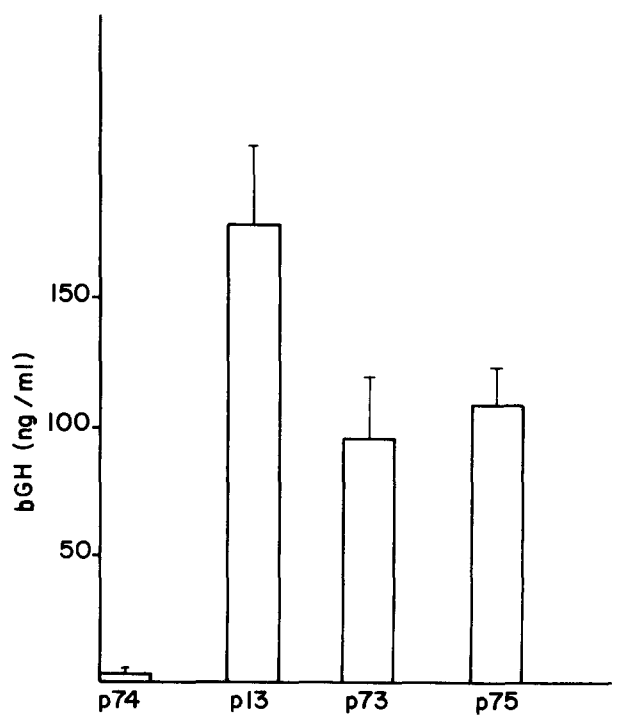

Fig. 4. The IRES effect of R regions from HTLV-1 and BLV in CHO cells. The gene constructs contained the hGH and bGH cDNAs driven by the hCMV promoter. bGH was measured in the medium 2 days after transfection.

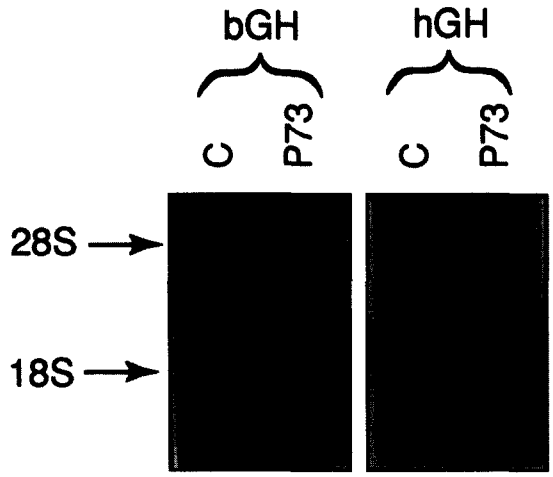

Fig. 5. Nothern blot analysis of the mRNA transcribed from the plasmid p73 transfected into CHO cells. Controls were non-transfected CHO cells (Fig. 1). Hybridizations were carried out using bGH and hGH cDNA probes.

data is that ribosomes were recruited by the SU 'R' sequence independently of the luciferase cistron.

4. Discussion

The data reported here leave little doubt on the fact that the R and the beginning of the U5 regions from HTLV-1 have the capacity to stimulate the translation of the second cistron in bicistronic mRNA. This adds to the list of the mRNAs having a 5'UTR which allows initiation of translation without a cap in its vicinity. A sequence containing an IRES activity has been found recently in two other retroviruses, the Moloney mouse leukaemia virus [12] and the Friend mouse leukaemia virus [13]. Interestingly, however, in these two viruses, the IRES is located after the U5 region between two initiator codons. The function of the IRES seems then to favour the utilization of both initiator codon more or less independently. In the case of HTLV-1, the IRES is in the 'R' region. The included short part of the U5 might be important for the IRES function. One reason inclines to think that this is indeed the case. In picornavirus, the end of the IRES section contains a polypyrimidine region with the consensus sequence UUUC

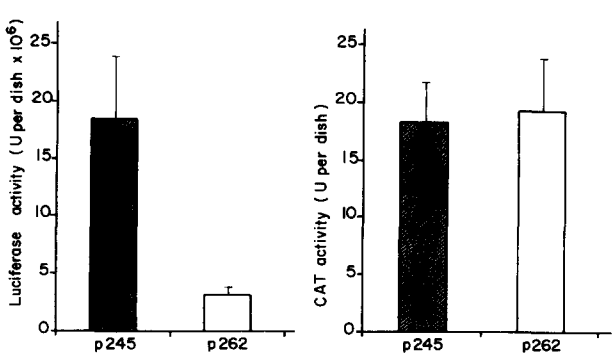

Fig. 6. The effect of a hairpin loop on the translation of the cistrons of a bicistronic mRNA containing the SU 'R' sequence. Plasmids p245 and p262 (Fig. 1) were transfected into CHO cells. Luciferase and CAT activities were measured in cell extracts. Results are the means ± SEM of three independent transfections.

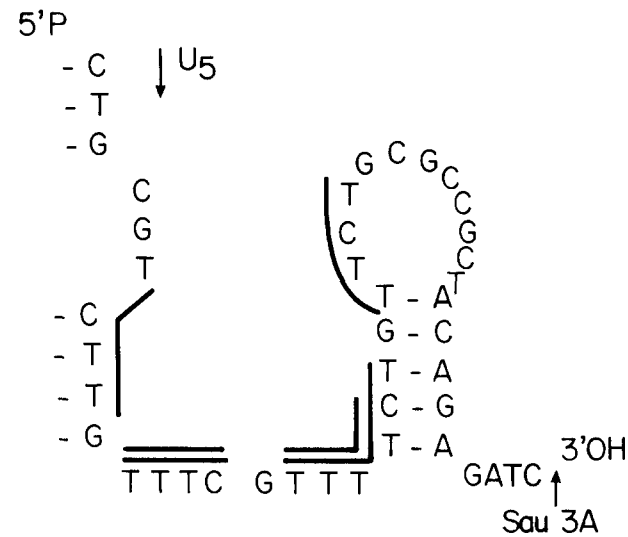

Fig. 7. Nucleotide sequence of the U5 region from HTLV-1 present in the gene constructs. The polypyrimidine regions are overlined, with two lines for the consensus sequence binding proteins.

or UUC [5,8]. It is noteworthy that such a polypyrimidine region was found in the Moloney mouse leukaemia virus [12] but also in the U5 part of the HTLV-1 sequences used in the present experiments (Fig. 7). The beginning of the U5 region of the BLV also contains a polypyrimidine sequence which might participate to the IRES activity [26]. This U5 region was not present in the BLV fragment used in the present experiment. This might explain why the R region from BLV showed a lower IRES activity than that from HTLV-1 (Figs. 2 and 3).

The exact role of the 5'UTR from SV40 is not known. In a previous study, we observed that this 5'UTR is highly structured and that it does not alter the secondary structure of the HTLV-1 'R' region when associated to it (Attal et al., unpublished results, [21]).

From a practical point of view, the IRES formed by the association of SU and 'R' from HTLV-1 described here offers several advantages to generate efficient bicistronic mRNAs. Indeed, the poliovirus IRES is not active in all cell type and its effect is maximum only in poliovirus infected cells. The encephalomyocarditis virus IRES seems less sensitive to the cell type used, but the AUG which is recognized as the initiator must be located in a precise position, a property which complicates gene construction. The new SU 'R' IRES described here is therefore highly flexible and efficient. For these reasons it may be preferred to others.

References

[1] Pain, V.M. (1996) Eur. J. Biochem. 236, 747–771.
[2] Kozak, M. (1995) Proc. Natl. Acad. Sci. USA 92, 2662–2666.
[3] Kozak, M. (1989) Mol. Cell. Biol. 9, 5134–5142.
[4] Sonenberg, N. (1991) TIG 7, 105–106.
[5] Sonenberg, N. (1994) Curr. Opin. Genet. Dev. 4, 310–315.
[6] Kozak, M. (1987) Mol. Cell. Biol. 7, 3438–3445.
[7] Mountford, P.S. and Smith, A.G. (1995) TIG 11, 179–184.
[8] Jackson, R.J., Howell, M.T. and Kaminski, A. (1990) TIBS 477–483.
[9] Rijnbrand, R., Bredenbeek, P., Van Der Straaten, T., Whetter, L., Inchauspe, G., Lemon, S. and Spaan, W. (1995) FEBS Lett. 365, 115–119.
[10] Thiel, V. and Siddell, S.G. (1994) J. Virol. 75, 3041–3046.

[11] Poole, T.L., Wang, C., Popp, R.A., Potgieter, L.N.D., Siddiqui, A. and Collett, M.S. (1995) Virology 206, 750–754.
[12] Vagner, S., Waysbort, A., Marenda, M., Gensac, M.C., Amalric F. and Prats, A.C. (1995) J. Biol. Chem. 270, 20376–20383.
[13] Berlioz, C. and Darlix, J.L. (1995) J. Virol. 69, 2214–2222.
[14] Lu, H., and Wimmer, E. (1996) Proc. Natl. Acad. Sci. USA 93, 1412–1417.
[15] Toyoda, H., Koide, N., Kamiyama, M., Tobita, K., Mizumoto, K. and Imura, N. (1994) Arch. Virol. 138, 1–15.
[16] Macejak, D.G. and Sarnow, P. (1991) Nature 353, 90–94.
[17] Oh, S.K., Scott, M.P. and Sarnow, P. (1992) Gens Dev. 6, 1643–1653.
[18] Chernajovsky, Y.L.A. (1995) UK Patent Application No. 942 41 30.4.
[19] Sarnow, P. (1989) Proc. Natl. Acad. Sci. USA 86, 5795–5799.
[20] Ohlmann, T., Rau, M., Pain, V.M. and Morley, S.J. (1996) EMBO J. 15, 1371–1382.
[21] Petitclerc, D., Attal, J., Théron, M.C., Bearzotti, M., Bolifraud, P., Kann, G., Stinnakre, M.G., Pointu, H., Puissant, C. and Houdebine, L.M. (1995) J. Biotech. 40, 169–178.
[22] Takebe, Y., Seiki, M., Fujisawa, J., Hoy, P., Yokota, K., Arai, K., Yoshida, M. and Arai, N. (1988) Mol. Cell. Biol. 8, 466–472.
[23] De Wet, J.R., Wood, K.V., De Luca, M., Helinski, D.R. and Subramani, S. (1987) Mol. Cell. Biol. 7, 725–737.
[24] Nielsen, D.A., Chang, T.S. and Shapiro, D.J. (1989) Anal. Biochem. 179, 19–23.
[25] Puissant, C. and Houdebine, L.M. (1990) Biotechniques 8, 148–149.
[26] Derse, D., Diniak, A.J., Casey, J.W. and Deininger, P.L. (1985) Virology 141, 162–166.